TABLE 47
Tests for Heterogeneity in Site-Specific Excess Absolute Rate Levels and Effect Modification Patterns

Site	EAR effect modification[a]			
	Age at exposure	Attained age	Gender	Global[c]
Oral cavity	0.12[b]	**0.04**[b]	0.40[b]	0.18[b]
Esophagus	0.08	>0.5	0.15	**0.10**
Stomach	0.21	>0.5	>0.5	0.41
Colon	>0.5	0.41	**0.02**	**0.002**
Rectum	>0.5	**0.07**	0.47	0.28
Liver	0.12	0.31	**0.06**	0.15
Gallbladder	>0.5	>0.5	>0.5	>0.5
Pancreas	0.37	>0.5	**0.15**	>0.5
Lung	**<0.001**	**<0.001**	0.20	**<0.001**
Skin	**<0.001**	**<0.001**	>0.5	**<0.001**
Breast	**0.002**	**0.002**	—	**0.003**
Bladder	**0.03**	**0.004**	0.32	**0.03**
Uterus	>0.5	>0.5	—	>0.5
Ovary	>0.5	>0.5	—	>0.5
Prostate	>0.5	>0.5	—	>0.5
Renal cell	0.33	0.17	>0.5	>0.5
CNS	**0.05**	**0.07**	**0.02**	**0.003**
Thyroid	>0.5	**0.004**	0.19	**0.004**
Other	>0.5	>0.5	0.13	0.38

[a] The effect modification tests are made assuming that the level can vary.

[b] Two-sided *P* values for tests of hypotheses that the solid cancer effect estimates apply to specific sites. *P* values of 0.1 or less are printed in bold.

[c] The global test is a test that all three (two for gender-specific cancers) parameters have the same value as those for solid cancer.

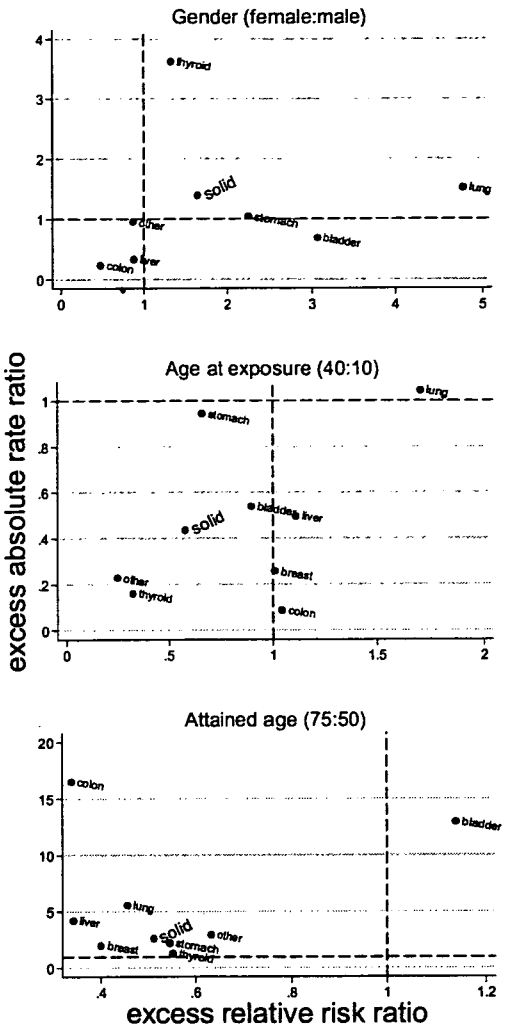

FIG. 23. Summary of site-specific excess relative risk (ERR) and excess absolute rate (EAR) effect modification for gender (top panel), age at exposure (middle panel), and attained age (bottom panel) for selected sites and all solid cancers. Plotting positions are determined by effect ratios in ERR (abscissa) and EAR (ordinate) models. Gender effects are defined as the ratio of the female risks to male risks. Age-at-exposure effects are defined as the ratio of the risk for exposure at age 40 to that for exposure at age 10. Attained-age effects are defined as the ratio of the risk at age 75 to the risk at age 50. The points for the other category are based on the results of analyses of the 5,396 cancer cases not included in the sites explicitly considered here. The dashed grid lines correspond to no variation in the ERR (vertical line) or EAR (horizontal line). Among other things, the plots highlights the extreme gender and age-at-exposure effects for lung and thyroid cancers and the unusual attained-age patterns for bladder and colon cancers.

solid cancer, while the lung cancer ERR appeared to increase more rapidly, with increasing age at exposure There were suggestions that changes in the ERR with attained age for bladder and renal cell cancers may be less rapid than those for all solid cancers. Gender differences in the ERR for cancers of colon, brain and central nervous system, and lung appeared to differ significantly from those seen for all solid cancers. The female:male ERR ratios for colon cancer and tumors of the brain and central nervous system were smaller than those for solid cancers as a group, while the female:male ERR ratio for lung cancer was considerably larger than that for all solid cancers. As noted previously, the large gender effect for lung cancer is primarily a consequence of confounding arising from the gender differences in smoking rates in Japan.

As shown in Table 47, solid cancer EAR effect modification parameters did not adequately describe effects for many sites.

Figure 23 contrasts the site-specific ratios of the ERRs (vertical axes) to the corresponding ratios for EARs for gender, age at exposure, and attained age. In the gender effect (upper) panel, thyroid cancer stands out as having an unusually large female:male EAR ratio, whereas the ERR ratio is similar to that for all solid cancers combined. For lung cancer, the ERR ratio is exceptional, suggesting that the gender difference in baseline rates may not completely explain the gender differences in lung cancer excess rates.

Looking at the panel for age at exposure (middle), we see that EARs are decreasing with increasing age at exposure (ratios less than 1) for all sites other than stomach and lung cancer, for which the EAR ratios are close to 1. Lung cancer had the highest ERR age-at-exposure effect (most likely, as noted earlier, due to the effect of smoking on lung cancer baseline rates). Thyroid cancer and the groups of

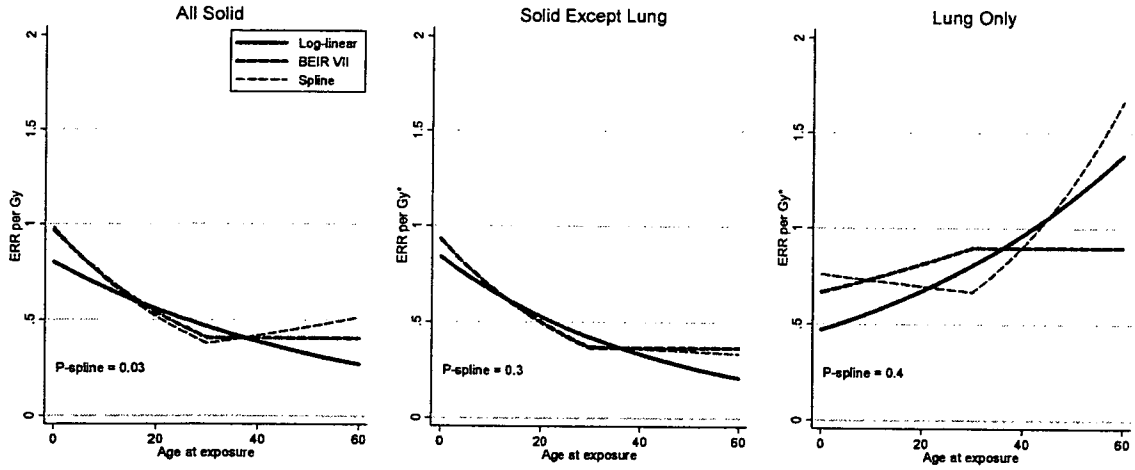

FIG. 24. Alternative age-at-exposure trends for all solid cancers, all solid cancers except lung cancer, and lung cancer. The solid line is the log-linear trend used in the models for this paper. The thick dashed line is a constrained spline with a log-linear trend for exposure ages prior to age 30 and no change after age 30. This is the form used for the BEIR VII risk models. The thin dashed line shows an unconstrained spline with a knot at age 30. The *P* values are based on likelihood ratio tests of the hypothesis that the unconstrained spline fits better than the log-linear trend used for most of the age-at-exposure effect modeling in this paper.

other cancers described above had striking age-at-exposure effects (as reflected by small ratios) for both the ERR and the EAR. EARs were estimated to be increasing with increasing attained age for all sites considered, with particularly large increases for colon and bladder cancers. Bladder cancer was the only site for which the estimated ERR increased with increasing attained age (albeit slightly). The most rapid declines in the ERR with attained age were seen for colon, liver and breast cancers.

The BEIR VII Models

The recently published National Research Council report on the biological effects of radiation, BEIR VII (*35*), made use of site-specific and all-solid-cancer risk models based on the data used for the analyses in this report. The BEIR VII models vary in several respects from those described in this report. In this section, we outline the differences and look at their impact on the nature of the fitted risk models.

The BEIR VII solid cancer models, which did not include the NIC group in the baseline risk modeling, were based on all solid cancers except thyroid and non-melanoma skin cancer. Parametric baseline rate models similar to those used in our ERR and EAR analyses were used in the BEIR VII models; however, the BEIR VII ERR baseline rates were described using a nonparametric model defined by stratification over city, gender, attained-age, and age-at-exposure categories. The use of stratified baseline models and the exclusion of the NIC have almost no impact on the fitted excess risks. The fitted ERRs for solid cancers other than thyroid and skin cancer are somewhat lower than those for all solid cancers, with the largest differences observed for childhood exposures.

The most important difference between the basic excess risk models used in this report and those used in BEIR VII concerns the handling of age-at-exposure effects on the excess risk (ERRs and EARs). In our analyses (see Eq. 1), excess risks were generally assumed to vary log-linearly with exposure age. However, as noted in our presentation of the results for all solid cancer, there were indications that standardized excess risks declined with age at exposure less than 40 years, while standardized risks for all solid cancers as a group increased with age at exposure for exposure late in life. A similar pattern was noted in the BEIR VII modeling, and as a result, in the final BEIR VII models [Tables 12-1 and 12-2 and Annex 12-B in ref. (*35*)] it was assumed that the excess risk varied as a constrained log-linear spline of age at exposure with a log-linear trend for ages 0 to 30 with no further change after age at exposure 30. The left panel in Fig. 24 contrasts the age-at-exposure effects for our log-linear trend model (solid line) with the BEIR VII constrained spline (thick dashed line) and an unconstrained spline with a knot at age 30. For exposures prior to age 20 or after age 40, the fitted ERRs from our standard model are lower than estimates from a BEIR VII-like model, while for those exposed between about 20 and 40 years of age, our model predicts slightly higher risks. The unconstrained spline model fits the all solid-cancer data significantly better than the simple log-linear trend ($P = 0.03$). A formal significance test to compare the BEIR VII constrained spline and the simple log-linear model is not possible, but the deviance reduction of 3.9 associated with the introduction of the constrained spline corresponds to a P value of 0.05 for a 1 *df* test.

As was noted above, the lung cancer ERR appears to increase with increasing age at exposure. Our analysis of the lung cancer data indicates that the unconstrained spline does not describe the lung cancer risks significantly better than the simple log-linear trend ($P = 0.4$) while the BEIR VII constrained spline fits slightly worse than the simple log-linear trend. These patterns can be seen in the rightmost

panel of Fig. 24. Analysis of the age-at-exposure trends for all solid cancers except lung cancer, as seen in the middle panel of Fig. 24, indicates that while the unconstrained spline shows a flattening for exposures after age 30, this does not offer a significant improvement over the simple trend ($P = 0.3$).

The suggestion that a more complex pattern provides a somewhat better fit to the data for all solid cancers as a group reflects to some extent the rather anomalous result for lung cancer. However, there are other sites, most notably stomach and liver, for which the data suggest that risks for those exposed late in life may be somewhat larger than predicted by the simple log-linear trend. In our opinion a simple log-linear trend provides an adequate, simple description of the age-at-exposure effects for most cancer sites, but in future analyses more attention should be given to alternative descriptions of age-at-exposure effects on both the ERR and EAR.

Main Conclusions and New Findings

More than 50 years after the atomic bombs were dropped on Hiroshima and Nagasaki, the updated solid cancer incidence data demonstrated that the shape of the dose response is well described by a linear no-threshold model. With the introduction of DS02, the risk estimates were generally reduced by about 10%, but the patterns of risk generally remained the same. The solid cancer EAR increased throughout life for all ages. The ERR decreased with increasing attained age; however, an elevated risk was still observed at the end of follow-up. The age-at-exposure effect is somewhat smaller in the current follow-up than in previous reports. Overall, women had higher excess relative and absolute risks than men, although when gender-specific cancers were excluded from the analyses the gender-specific excess absolute rates were essentially equal.

The large number of additional cancers in the current follow-up provided more precise estimates of risk and allowed us to conduct more detailed analyses of the effects of gender, age and time. Thus we found that the elevated radiation-associated relative and absolute risks of esophageal cancer now reached statistical significance and that the ERR for cancer of the uterine corpus among women exposed during the bombings before age 20 years was increased, although the point estimate was of borderline statistical significance.

Another new finding is the significant dose response observed for each of the five main histological groupings (adenocarinomas, squamous cell carcinomas, other and unclassified epithelial cancers, sarcomas, and other non-epithelial cancers). Sarcomas, as a group, were of particular interest because information on radiation exposure and the risk of sarcomas is sparse and previous reported associations usually were observed after high-dose radiotherapy (*127*).

The additional years of follow-up allowed a more precise evaluation of radiation-related cancer risks for those cancers that generally develop late in life. Thus the large ERR noted for bladder cancer in this report emphasizes the radiosensitivity of the bladder at all ages at exposure and demonstrates, for the first time, an elevated ERR among persons exposed to the bombings before age 20. Additional follow-up should help clarify the link between radiation exposure and cancers occurring among the elderly, such as gallbladder, pancreas and prostate cancers, for which associations have not been seen to date.

Finally, we described the large relative increases in the risk of occurrence of adolescent and young adult malignancies associated with childhood radiation exposure. Subset analyses based solely on cases diagnosed prior to ages 20, 25 and 30 suggest that the ERR for solid cancers diagnosed before age 13, i.e. prior to the beginning of this follow-up, would have been extremely large. Since baseline cancer rates are very low at young ages, the excess absolute rates (and number of excess cases) would not be large.

Predictions for the Future

While a great deal has already been learned about radiation risks from the LSS, important questions about age and temporal patterns remain. We expect that over the next 15 to 20 years, a large proportion of the radiation-associated excess solid cancers will be diagnosed. Based on a linear dose–response model that allows for changes in the ERR with attained age and age at exposure, it is predicted that the peak number of cancers per year will be reached in about 2015. The accumulating data should therefore offer important new insights into lifetime risks, risk patterns for survivors who were less than 20 years old at the time of the bombings, and gender differences in risks. Since children continue to be exposed to radiation from medical diagnostic X-ray examinations, interventional radiology, and radiotherapy, further follow-up of the LSS is essential to understanding the lifetime cancer risks associated with exposure to radiation in childhood or adolescence. Indeed, the LSS is the single largest cohort of generally healthy individuals exposed to radiation as children. Ongoing site-specific incidence studies of cancers of the thyroid, lung and ovary as well as lymphoma, which include detailed pathological reviews of tumor tissue, should advance our knowledge regarding radiation effects for specific histological types of cancer. Future studies focusing on uterine cancers (corpus vs. cervix) and sarcomas of different organs and tissues may also provide new information on the radiation effects. With close collaboration among epidemiologists, statisticians, radiobiologists and pathologists, we should be able to improve our understanding of radiation risks and their implications for radiation protection.

APPENDIX

This appendix contains tables that give information on the distribution of cases and crude rates stratified by age at exposure (birth cohort), dose and gender for each of the sites considered in the main text of this report. For relatively less common cancer types, three age-at-exposure groups are used (0–19, 20–39 and 40+) while for sites with more cases, there are six age-at-exposure groups (0–9, 10–19, 20–29, 30–39, 40–49 and 50+). Although these tables provide useful simple summaries of the data, it should be kept in mind that birth cohort and age at exposure are perfectly correlated in this cohort and that lifetime follow-up is far from complete for those who were under age 40 at the time of exposure (i.e. born after 1905). Thus the increased risks in older age-at-exposure (earlier year of birth) cohorts reflect differences in the range of follow-up ages and cannot readily be interpreted as reflecting birth cohort effects on the baseline rates.

The detailed person-year table used for these analyses is available through the RERF home page (www.rerf.jp).

TABLE A1
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancers of the Oral Cavity and Pharynx by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	1.0	1.2	0.9	3.1	0.4	0.4	1.0	1.8
	Cases	37	27	2	7	16	9	3	5
20–39	Crude rate	2.2	2.1	2.2	2.0	0.7	0.6	0.6	1.8
	Cases	30	15	2	2	29	14	2	4
40+	Crude rate	2.4	2.5	2.7	1.4	0.9	1.3	0.8	—
	Cases	25	15	2	1	15	14	1	—

[a] Weighted skin dose (shielded kerma) in Gy.

TABLE A2
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancer of the Esophagus by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	1.3	1.1	1.8	4.1	0.1	0.1	—	0.7
	Cases	47	27	3	6	5	3	—	1
20–39	Crude rate	3.2	3.4	5.6	1.8	0.5	0.5	—	—
	Cases	45	26	4	1	18	13	—	—
40+	Crude rate	7.0	7.1	3.6	10.6	0.8	1.0	2.4	5.0
	Cases	72	47	2	4	13	11	2	2

[a] Weighted stomach dose in Gy.

TABLE A3
Crude Stomach Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	5.0	5.6	4.3	17.1	2.6	2.7	6.2	8.7
	Cases	91	74	3	10	52	39	5	5
10–19	Crude rate	13.2	15.1	18.2	17.3	5.5	5.9	8.9	11.7
	Cases	236	161	18	15	134	80	14	11
20–29	Crude rate	30.1	25.6	43.7	35.0	9.9	12.3	12.3	25.7
	Cases	180	85	13	9	211	162	15	18
30–39	Crude rate	43.2	42.8	45.6	54.2	18.1	20.2	17.6	31.7
	Cases	343	190	19	16	322	252	13	17
40–19	Crude rate	59.9	52.5	63.7	83.2	22.8	24.0	21.2	34.8
	Cases	424	231	24	22	260	189	13	11
50+	Crude rate	63.9	78.5	63.9	96.1	35.3	42.8	46.8	80.0
	Cases	207	172	11	11	175	150	10	7

[a] Weighted stomach dose in Gy.

TABLE A4
Crude Colon Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	3.2	2.3	1.4	10.3	1.5	1.6	3.7	5.2
	Cases	58	31	1	6	29	23	3	3
10–19	Crude rate	6.8	7.9	14.2	19.7	3.9	2.8	3.2	4.3
	Cases	122	84	14	17	94	38	5	4
20–29	Crude rate	10.2	10.9	9.9	7.9	5.9	6.1	5.7	14.3
	Cases	61	36	3	2	125	80	7	10
30–39	Crude rate	7.3	12.4	4.8	27.5	6.6	7.4	4.1	5.6
	Cases	58	55	2	8	117	92	3	3
40–49	Crude rate	7.2	8.6	16.0	7.6	6.9	6.0	4.8	9.7
	Cases	51	38	6	2	79	47	3	3
50+	Crude rate	6.2	7.3	11.4	0.0	6.3	5.7	18.7	0.0
	Cases	20	16	2	0	31	20	4	0

[a] Weighted colon dose in Gy.

TABLE A5
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancer of the Rectum by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	2.7	2.3	4.1	3.4	1.4	1.4	1.3	3.3
	Cases	98	54	7	5	60	40	3	5
20–39	Crude rate	5.4	6.1	2.8	5.5	2.7	3.4	4.1	4.9
	Cases	75	47	2	3	105	87	8	6
40+	Crude rate	6.6	7.9	9.1	5.3	3.4	3.8	6.0	5.1
	Cases	68	52	5	2	56	43	5	2

[a] Weighted bladder dose in Gy.

TABLE A6
Crude Liver Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	2.7	2.6	1.4	8.5	0.4	0.3	0.0	1.7
	Cases	50	34	1	5	7	4	0	1
10–19	Crude rate	9.8	12.8	16.2	16.2	2.9	2.7	3.2	6.4
	Cases	176	137	16	14	71	37	5	6
20–29	Crude rate	10.4	10.6	33.3	18.8	3.9	3.7	3.3	4.3
	Cases	62	35	10	5	83	49	4	3
30–39	Crude rate	11.8	10.4	5.0	15.9	5.5	4.7	2.8	3.6
	Cases	94	46	2	5	98	58	2	2
40–49	Crude rate	11.4	11.1	10.7	0.0	5.5	6.6	14.5	3.2
	Cases	81	49	4	0	63	52	9	1
50+	Crude rate	12.0	10.5	28.7	17.2	5.0	4.3	14.0	11.2
	Cases	39	23	5	2	25	15	3	1

[a] Weighted liver dose in Gy.

TABLE A7
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancer of the Gallbladder and Extrahepatic Bile Ducts by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	2.7	2.3	4.1	3.4	1.4	1.4	1.3	3.3
	Cases	98	54	7	5	60	40	3	5
20–39	Crude rate	5.4	6.1	2.8	5.5	2.7	3.4	4.1	4.9
	Cases	75	47	2	3	105	87	8	6
40+	Crude rate	6.6	7.9	9.1	5.3	3.4	3.8	6.0	5.1
	Cases	68	52	5	2	56	43	5	2

[a] Weighted pancreas dose in Gy.

TABLE A8
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancer of the Pancreas by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	1.0	0.7	0.6	0.7	0.3	0.4	1.7	2.0
	Cases	35	17	1	1	14	12	4	3
20–39	Crude rate	2.4	3.7	8.1	0.0	2.2	1.8	3.6	4.1
	Cases	34	29	6	0	88	47	7	5
40+	Crude rate	5.3	5.6	5.4	13.5	3.5	4.2	4.7	0.0
	Cases	55	37	3	5	57	48	4	0

[a] Weighted pancreas dose in Gy.

TABLE A9
Crude Lung Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	1.4	1.2	0.0	3.4	0.7	0.6	2.5	1.7
	Cases	26	16	0	2	13	9	2	1
10–19	Crude rate	5.2	6.3	8.3	7.9	1.8	1.3	2.7	5.8
	Cases	93	67	7	8	44	18	4	6
20–29	Crude rate	12.7	9.4	11.8	12.4	3.7	4.6	5.4	13.7
	Cases	76	31	3	4	80	60	6	11
30–39	Crude rate	20.0	17.4	7.9	34.3	5.3	7.4	10.9	15.9
	Cases	159	77	3	12	95	92	7	10
40–49	Crude rate	25.4	24.5	41.8	27.9	7.4	8.9	9.9	28.3
	Cases	180	108	15	8	84	70	5	12
50+	Crude rate	23.5	23.8	49.6	52.7	8.9	11.7	24.9	18.4
	Cases	8	6	1	0	23	22	1	1

[a] Weighted lung dose in Gy.

TABLE A10
Crude Non-melanoma Skin Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.0	0.3	0.0	3.8	0.1	0.0	0.7	6.7
	Cases	0	4	0	3	1	0	1	6
10–19	Crude rate	0.7	1.0	0.0	6.2	0.2	0.2	0.6	2.0
	Cases	12	10	0	9	5	3	1	4
20–29	Crude rate	1.0	1.3	0.0	9.5	0.7	0.7	1.2	3.9
	Cases	6	4	0	4	16	9	2	5
30–39	Crude rate	2.1	1.5	2.0	0.0	1.9	2.6	1.5	4.2
	Cases	17	6	1	0	33	30	2	4
40–49	Crude rate	2.1	3.2	4.4	3.7	3.0	2.4	3.6	5.2
	Cases	15	13	2	2	34	17	3	4
50+	Crude rate	2.2	3.5	3.6	0.0	3.4	2.2	4.9	5.7
	Cases	7	7	1	0	17	7	2	1

[a] Weighted skin dose (shielded kerma) in Gy.

TABLE A11
Crude Female Breast Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Weighted breast dose (Gy)			
		<0.005	−0.5	−1	1–4
0–9	Crude rate	4.3	5.1	8.8	20.9
	Cases	85	72	5	17
10–19	Crude rate	6.1	7.2	13.6	25.9
	Cases	149	95	17	40
20–29	Crude rate	6.1	7.6	11.5	16.6
	Cases	130	97	14	18
30–39	Crude rate	5.5	5.6	8.7	22.6
	Cases	98	67	10	16
40–49	Crude rate	5.4	4.9	5.9	8.4
	Cases	62	37	5	4
50+	Crude rate	4.4	2.7	7.0	14.8
	Cases	22	9	2	2

TABLE A12
Crude Uterine Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Weighted uterine dose (Gy)			
		<0.005	−0.5	−1	1–4
0–9	Crude rate	4.1	4.0	3.8	7.3
	Cases	180	110	9	11
10–19	Crude rate	7.6	8.1	7.0	7.5
	Cases	298	207	14	9
20–29	Crude rate	11.0	11.7	8.4	10.2
	Cases	180	133	7	4
30–39	Crude rate	4.1	4.0	3.8	7.3
	Cases	180	110	9	11
40–49	Crude rate	7.6	8.1	7.0	7.5
	Cases	298	207	14	9
50+	Crude rate	11.0	11.7	8.4	10.2
	Cases	180	133	7	4

TABLE A13
Crude Ovarian Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Weighted ovarian dose (Gy)			
		<0.005	−0.5	−1	1–4
0–9	Crude rate	1.0	0.8	1.3	3.3
	Cases	43	21	2	6
10–19	Crude rate	1.6	1.5	1.5	2.5
	Cases	61	38	4	3
20–29	Crude rate	2.3	2.1	3.5	5.2
	Cases	38	22	4	3
30–39	Crude rate	1.0	0.8	1.3	3.3
	Cases	43	21	2	6
40–49	Crude rate	1.6	1.5	1.5	2.5
	Cases	61	38	4	3
50+	Crude rate	2.3	2.1	3.5	5.2
	Cases	38	22	4	3

TABLE A14
Crude Prostate Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Weighted bladder dose (Gy)			
		<0.005	−0.5	−1	1–4
0–9	Crude rate	0.8	0.6	0.6	2.1
	Cases	28	14	1	3
10–19	Crude rate	6.7	6.3	9.7	5.5
	Cases	94	49	7	3
20–29	Crude rate	10.6	10.8	9.1	7.9
	Cases	109	71	5	3
30–39	Crude rate	0.8	0.6	0.6	2.1
	Cases	28	14	1	3
40–49	Crude rate	6.7	6.3	9.7	5.5
	Cases	94	49	7	3
50+	Crude rate	10.6	10.8	9.1	7.9
	Cases	109	71	5	3

TABLE A15
Crude Incidence Rates (Cases per 10,000 Person Years) for Renal Cell Cancer by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.4	0.4	0.0	2.1	0.3	0.3	0.4	1.3
	Cases	16	9	0	3	12	8	1	2
20–39	Crude rate	0.9	1.3	0.0	0.0	0.4	0.5	1.0	0.8
	Cases	13	10	0	0	15	14	2	1
40+	Crude rate	2.7	1.8	0.0	0.0	0.8	0.7	0.0	0.0
	Cases	28	12	0	0	13	8	0	0

[a] Weighted bladder dose in Gy.

TABLE A16
Crude Bladder Cancer Incidence Rates (Cases per 10,000 Person Years) by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.8	0.9	0.0	1.7	0.1	0.2	0.0	0.0
	Cases	15	12	0	1	2	3	0	0
10–19	Crude rate	2.0	1.8	2.0	5.8	0.3	0.4	0.6	0.0
	Cases	35	19	2	5	7	5	1	0
20–29	Crude rate	3.8	3.6	6.7	15.6	0.7	1.6	2.5	1.4
	Cases	23	12	2	4	16	21	3	1
30–39	Crude rate	4.8	3.4	0.0	0.0	0.7	1.5	1.3	3.8
	Cases	38	15	0	0	12	19	1	2
40–49	Crude rate	5.2	6.8	16.0	11.3	1.5	2.0	1.6	6.5
	Cases	37	30	6	3	17	16	1	2
50+	Crude rate	9.9	8.7	5.7	8.8	2.6	3.1	13.8	11.8
	Cases	32	19	1	1	13	11	3	1

[a] Weighted bladder dose in Gy.

TABLE A17
Crude Incidence Rates (Cases per 10,000 Person Years) for Tumors of the Brain and Central Nervous System by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.7	0.7	1.5	4.9	0.9	0.7	1.1	1.0
	Cases	25	16	2	9	39	19	2	2
20–39	Crude rate	1.0	0.5	3.6	1.2	0.8	1.3	2.1	2.2
	Cases	14	4	2	1	32	34	3	4
40+	Crude rate	0.9	1.2	9.3	0.0	1.5	2.2	2.8	0.0
	Cases	9	8	4	0	25	25	2	0

[a] Weighted brain dose in Gy.

TABLE A18
Crude Incidence Rates (Cases per 10,000 Person Years) for Thyroid Cancer by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.4	0.6	0.7	3.9	1.3	2.0	5.4	8.8
	Cases	13	14	1	7	57	55	10	18
20–39	Crude rate	0.7	0.5	5.8	1.2	1.4	2.6	4.0	3.8
	Cases	10	4	3	1	54	66	6	7
40+	Crude rate	1.8	2.6	2.1	0.0	3.2	4.1	4.2	9.8
	Cases	19	17	1	0	53	46	3	6

[a] Weighted thyroid dose in Gy.

TABLE A19
Crude Incidence Rates (Cases per 10,000 Person Years) for Cancers not Considered in Other Analyses by Age-at-Exposure and Dose Categories (in Gy)

Age at exposure (years)		Male				Female			
		<0.005[a]	−0.5	−1	1–4	<0.005	−0.5	−1	1–4
0–9	Crude rate	0.7	1.1	1.4	6.8	0.3	0.5	2.5	5.2
	Cases	12	15	1	4	5	7	2	3
10–19	Crude rate	2.4	2.3	4.0	5.8	0.9	1.2	2.6	3.2
	Cases	43	25	4	5	22	16	4	3
20–29	Crude rate	3.8	4.8	9.9	4.0	2.2	2.2	2.5	7.1
	Cases	23	16	3	1	46	29	3	5
30–39	Crude rate	6.2	7.2	16.6	17.2	3.4	3.9	6.8	3.7
	Cases	49	32	7	5	60	48	5	2
40–49	Crude rate	7.9	8.6	5.3	7.6	4.3	6.1	6.4	13.0
	Cases	56	38	2	2	49	48	4	4
50+	Crude rate	11.4	11.0	5.7	26.5	6.1	5.1	4.7	23.2
	Cases	37	24	1	3	30	18	1	2

[a] Weighted colon dose in Gy.

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REFERENCES

1. K. Mabuchi, M. Soda, E. Ron, M. Tokunaga, S. Ochikubo, S. Sugimoto, T. Ikeda, M. Terasaki, D. L. Preston and D. E. Thompson, Cancer incidence in atomic bomb survivors. Part I: Use of the tumor registries in Hiroshima and Nagasaki for incidence studies. *Radiat. Res.* **137** (Suppl.), S1–S16 (1994).
2. D. E. Thompson, K. Mabuchi, E. Ron, M. Soda, M. Tokunaga, S. Ochikubo, S. Sugimoto, T. Ikeda, M. Terasaki and D. L. Preston, Cancer incidence in atomic bomb survivors. Part II: Solid tumors, 1958–1987. *Radiat. Res.* **137** (Suppl.), S17–S67 (1994).
3. D. L. Preston, S. Kusumi, M. Tomonaga, S. Izumi, E. Ron, A. Kuramoto, N. Kamada, H. Dohy, T. Matsuo and K. Mabuchi, Cancer incidence in atomic bomb survivors. Part III. Leukemia, lymphoma and multiple myeloma, 1950–1987. *Radiat. Res.* **137** (Suppl.), S68–S97 (1994).
4. E. Ron, D. L. Preston, K. Mabuchi, D. E. Thompson and M. Soda, Cancer incidence in atomic bomb survivors. Part IV: Comparison of cancer incidence and mortality. *Radiat. Res.* **137** (Suppl.), S98–S112 (1994); Erratum, *Radiat. Res.* **139**, 129 (1994).
5. D. A. Pierce and D. L. Preston, Radiation-related cancer risks at low doses among atomic bomb survivors. *Radiat. Res.* **154**, 178–186 (2000).
6. E. Ron, D. L. Preston, M. Kishikawa, T. Kobuke, M. Iseki, S. Tokuoka, M. Tokunaga and K. Mabuchi, Skin tumor risk among atomic-bomb survivors in Japan. *Cancer Causes Control* **9**, 393–401 (1998).
7. C. E. Land, T. Saku, Y. Hayashi, O. Takahara, H. Matsuura, S. Tokuoka, M. Tokunaga and K. Mabuchi, Incidence of salivary gland tumors among atomic bomb survivors, 1950–1987. Evaluation of radiation-related risk. *Radiat. Res.* **146**, 28–36 (1996); Erratum, *Radiat. Res.* **146**, 356 (1996).
8. C. E. Land, M. Tokunaga, K. Koyama, M. Soda, D. L. Preston, I. Nishimori and S. Tokuoka, Incidence of female breast cancer among atomic bomb survivors, Hiroshima and Nagasaki, 1950–1990. *Radiat. Res.* **160**, 707–717 (2003).
9. J. B. Cologne, S. Tokuoka, G. W. Beebe, T. Fukuhara and K. Ma-

buchi, Effects of radiation on incidence of primary liver cancer among atomic bomb survivors. *Radiat. Res.* **152**, 364–373 (1999).

10. G. B. Sharp, J. B. Cologne, T. Fukuhara, H. Itakura, M. Yamamoto and S. Tokuoka, Temporal changes in liver cancer incidence rates in Japan: Accounting for death certificate inaccuracies and improving diagnostic techniques. *Int. J. Cancer* **93**, 751–758 (2001).
11. T. Fukuhara, G. B. Sharp, T. Mizuno, H. Itakura, M. Yamamoto, M. Tokunaga, S. Tokuoka, J. B. Cologne, Y. Fujita and K. Mabuchi, Liver cancer in atomic-bomb survivors: histological characteristics and relationships to radiation and hepatitis B and C viruses. *J. Radiat. Res. (Tokyo)* **42**, 117–130 (2001).
12. D. L. Preston, A. Mattsson, E. Holmberg, R. Shore, N. G. Hildreth and J. D. Boice, Jr., Radiation effects on breast cancer risk: A pooled analysis of eight cohorts. *Radiat. Res.* **158**, 220–235 (2002).
13. D. A. Pierce, G. B. Sharp and K. Mabuchi, Joint effects of radiation and smoking on lung cancer risk among atomic bomb survivors. *Radiat. Res.* **159**, 511–520 (2003).
14. D. L. Preston, E. Ron, S. Yonehara, T. Kobuke, H. Fujii, M. Kishikawa, M. Tokunaga, S. Tokuoka and K. Mabuchi, Tumors of the nervous system and pituitary gland associated with atomic bomb radiation exposure. *J. Natl. Cancer Inst.* **94**, 1555–1563 (2002).
15. S. Yonehara, A. V. Brenner, M. Kishikawa, P. D. Inskip, D. L. Preston, E. Ron, K. Mabuchi and S. Tokuoka, Clinical and epidemiologic characteristics of first primary tumors of the central nervous system and related organs among atomic bomb survivors in Hiroshima and Nagasaki, 1958–1995. *Cancer* **101**, 1644–1654 (2004).
16. E. Ron, T. Ikeda, D. L. Preston and S. Tokuoka, Male breast cancer incidence among atomic bomb survivors. *J. Natl. Cancer Inst.* **97**, 603–605 (2005).
17. M. Kishikawa, K. Koyama, M. Iseki, T. Kobuke, S. Yonehara, M. Soda, E. Ron, M. Tokunaga, D. L. Preston and S. Tokuoka, Histologic characteristics of skin cancer in Hiroshima and Nagasaki: Background incidence and radiation effects. *Int. J. Cancer* **117**, 363–369 (2005).
18. R. W. Young and G. D. Kerr, Eds., *Reassessment of the Atomic-Bomb Radiation Dosimetry for Hiroshima and Nagasaki: Dosimetry System 2002.* Radiation Effects Research Foundation, Hiroshima, 2005.
19. D. L. Preston, D. A. Pierce, Y. Shimizu, H. M. Cullings, S. Fujita, S. Funamoto and K. Kodama, Effect of recent changes in atomic bomb survivor dosimetry on cancer mortality risk estimates. *Radiat. Res.* **162**, 377–389 (2004).
20. D. L. Preston, Y. Shimizu, D. A. Pierce, A. Suyama and K. Mabuchi, Studies of mortality of atomic bomb survivors. Report 13: Solid 0cancer and noncancer disease mortality: 1950–1997. *Radiat. Res.* **160**, 381–407 (2003).
21. D. A. Pierce, Y. Shimizu, D. L. Preston, M. Vaeth and K. Mabuchi, Studies of the mortality of atomic bomb survivors. Report 12, Part I. Cancer: 1950–1990. *Radiat. Res.* **146**, 1–27 (1996).
22. Y. Shimizu, D. A. Pierce, D. L. Preston and K. Mabuchi, Studies of the mortality of atomic bomb survivors. Report 12, part II. Noncancer mortality: 1950–1990. *Radiat. Res.* **152**, 374–389 (1999).
23. J. B. Cologne and D. L. Preston, Longevity of atomic-bomb survivors. *Lancet* **356**, 303–307 (2000).
24. WHO, *International Classification of Diseases for Oncology (ICD-O-3).* World Health Organization, Geneva, 2000.
25. R. Sposto and D. L. Preston, *Correcting for Catchment Area Nonresidency in Studies Based on Tumor Registry Data.* Radiation Effects Research Foundation, Hiroshima, 1992.
26. ICRP, *1990 Recommendations of the International Commission on Radiological Protection.* Publication 60, *Annals of the ICRP,* Vol. 21, No. 1–3, Pergamon Press, Oxford, 1991
27. ICRU, *Quantities and Units in Radiation Protection Dosimetry.* Report 51, International Commission on Radiation Units and Measurements, Bethesda, MD, 1993.
28. D. A. Pierce, D. O. Stram and M. Vaeth, Allowing for random errors in radiation dose estimates for the atomic bomb survivor data. *Radiat. Res.* **123**, 275–284 (1990).
29. H. M. Cullings, S. Fujita, S. Funamoto, E. J. Grant, G. D. Kerr and D. L. Preston, Dose estimation for atomic bomb survivor studies: Its evolution and present status. *Radiat. Res.* **166**, 219–254 (2006).
30. D. Clayton and M. Hills, *Statistical Methods in Epidemiology.* Oxford University Press, New York, 1993.
31. N. E. Breslow and N. E. Day, *The Design and Analysis of Cohort Studies.* International Agency for Research on Cancer, Lyon, 1988.
32. D. L. Preston, J. H. Lubin, D. A. Pierce and M. E. McConney, *Epicure Users Guide.* Hirosoft International Corporation, Seattle, WA, 1993.
33. W. Ajiki, H. Tsukuma and A. Oshima, Cancer incidence and incidence rates in Japan in 1999: Estimates based on data from 11 population-based cancer registries. *Jpn. J. Clin. Oncol.* **34**, 352–356 (2004).
34. D. M. Parkin, S. L. Whelan, J. Ferlay, L. Teppo and D. B. Thomas, Eds., *Cancer Incidence in Five Continents, Vol. VIII.* IARC Scientific Publications No. 155, IARC, Lyon, 2002.
35. National Academy of Sciences, Committee on the Biological Effects of Radiation, *Health Risks From Exposure to Low Levels of Ionizing Radiation: BEIR VII Phase 2.* National Academies Press, Washington, DC, 2005.
36. NCRP, *Uncertainties in Fatal Cancer Risk Estimates Used in Radiation Protection.* Report 126, National Council on Radiation Protection and Measurements, Bethesda, MD, 1997.
37. UNSCEAR, *Sources and Effects of Ionizing Radiation: 2000 Report to the General Assembly, with Scientific Annexes, Vol. II: Effects.* United Nations, New York, 2000.
38. Research group for population-based cancer registration in Japan, Cancer incidence and incidence rates in Japan in 1998: Estimates based on data from 12 population-based cancer registries. *Jpn. J. Clin. Oncol.* **33**, 241–245 (2003).
39. E. Petridou, A. I. Zavras, D. Lefatzis, N. Dessypris, G. Laskaris, G. Dokianakis, J. Segas, C. W. Douglas, S. R. Diehl and D. Trichopoulos, The role of diet and specific micronutrients in the etiology of oral carcinoma. *Cancer* **94**, 2981–2988 (2002).
40. W. J. Blot, J. K. McLaughlin, D. M. Winn, D. F. Austin, R. S. Greenberg, S. Preston-Martin, L. Bernstein, J. B. Schoenberg, A. Stemhagen and J. F. Fraumeni, Jr., Smoking and drinking in relation to oral and pharyngeal cancer. *Cancer Res.* **48**, 3282–3287 (1988).
41. W. J. Blot, J. K. McLaughlin, S. S. Devesa and J. F. Fraumeni, Jr., Cancers of the oral cavity and pharynx. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 666–680. Oxford University Press, New York, 1996.
42. M. R. Spitz, B. C. Tilley, J. G. Batsakis, J. M. Gibeau and G. R. Newell, Risk factors for major salivary gland carcinoma. A case-comparison study. *Cancer* **54**, 1854–1859 (1984).
43. S. Preston-Martin, D. C. Thomas, S. C. White and D. Cohen, Prior exposure to medical and dental x-rays related to tumors of the parotid gland. *J. Natl. Cancer Inst.* **80**, 943–949 (1988).
44. J. L. Belsky, N. Takeichi, T. Yamamoto, R. W. Cihak, F. Hirose, H. Ezaki, S. Inoue and W. J. Blot, Salivary gland neoplasms following atomic radiation: Additional cases and reanalysis of combined data in a fixed population, 1957–1970. *Cancer* **35**, 555–559 (1975).
45. T. Saku, Y. Hayashi, O. Takahara, H. Matsuura, M. Tokunaga, M. Tokunaga, S. Tokuoka, M. Soda, K. Mabuchi and C. E. Land, Salivary gland tumors among atomic bomb survivors, 1950–1987. *Cancer* **79**, 1465–1475 (1997).
46. J. Lagergren, Adenocarcinoma of oesophagus: What exactly is the size of the problem and who is at risk? *Gut* **54** (Suppl. 1), i1–i5 (2005).
47. U. Ribeiro, Jr., A. V. Safatle-Ribeiro, M. R. Clarke and M. C. Posner, Primary malignant melanoma of the esophagus. *Am. J. Gastroenterol.* **91**, 1048–1049 (1996).
48. L. M. Brown and S. S. Devesa, Epidemiologic trends in esophageal and gastric cancer in the United States. *Surg. Oncol. Clin. N. Am.* **11**, 235–256 (2002).
49. H. Tsukuma, W. Ajiki, A. Ioka and A. Oshima, Survival of cancer patients diagnosed between 1993 and 1996: A collaborative study

of population-based cancer registries in Japan. *Jpn. J. Clin. Oncol.* **36**, 602–607 (2006).

50. L. S. Engel, W. H. Chow, T. L. Vaughan, M. D. Gammon, H. A. Risch, J. L. Stanford, J. B. Schoenberg, S. T. Mayne, R. Dubrow and J. F. Fraumeni, Jr., Population attributable risks of esophageal and gastric cancers. *J. Natl. Cancer Inst.* **95**, 1404–1413 (2003).
51. M. P. Little, Cancer after exposure to radiation in the course of treatment for benign and malignant disease. *Lancet Oncol.* **2**, 212–220 (2001).
52. L. B. Zablotska, A. Chak, A. Das and A. I. Neugut, Increased risk of squamous cell esophageal cancer after adjuvant radiation therapy for primary breast cancer. *Am. J. Epidemiol.* **161**, 330–337 (2005).
53. P. Correa, Bacterial infections as a cause of cancer. *J. Natl. Cancer Inst.* **95**, E3 (2003).
54. T. Kobayashi, S. Kikuchi, Y. Lin, K. Yagyu, Y. Obata, A. Ogihara, A. Hasegawa, K. Miki, E. Kaneko and H. Tenjin, Trends in the incidence of gastric cancer in Japan and their associations with Helicobacter pylori infection and gastric mucosal atrophy. *Gastric Cancer* **7**, 233–239 (2004).
55. Z. A. Carr, R. A. Kleinerman, M. Stovall, R. M. Weinstock, M. L. Griem and C. E. Land, Malignant neoplasms after radiation therapy for peptic ulcer. *Radiat. Res.* **157**, 668–677 (2002).
56. J. D. Boice, Jr., G. Engholm, R. A. Kleinerman, M. Blettner, M. Stovall, H. Lisco, W. C. Moloney, D. F. Austin, A. Bosch and B. McMahon, Radiation dose and second cancer risk in patients treated for cancer of the cervix. *Radiat. Res.* **116**, 3–55 (1988).
57. A. M. Nomura, Stomach cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 707–724. Oxford University Press, New York, 1996.
58. J. R. Kelley and J. M. Duggan, Gastric cancer epidemiology and risk factors. *J. Clin. Epidemiol.* **56**, 1–9 (2003).
59. S. Nakaji, T. Umeda, T. Shimoyama, K. Sugawara, K. Tamura, S. Fukuda, J. Sakamoto and S. Parodi, Environmental factors affect colon carcinoma and rectal carcinoma in men and women differently. *Int. J. Colorectal Dis.* **18**, 481–486 (2003).
60. S. Kono, Secular trend of colon cancer incidence and mortality in relation to fat and meat intake in Japan. *Eur J. Cancer Prev.* **13**, 127–132 (2004).
61. H. Y. Yiu, A. S. Whittemore and A. Shibata, Increasing colorectal cancer incidence rates in Japan. *Int. J. Cancer* **109**, 777–781 (2004).
62. P. D. Inskip, R. R. Monson, J. K. Wagoner, M. Stovall, F. G. Davis, R. A. Kleinerman and J. D. Boice, Jr., Cancer mortality following radium treatment for uterine bleeding. *Radiat. Res.* **123**, 331–344 (1990).
63. M. Lundell and L. E. Holm, Risk of solid tumors after irradiation in infancy. *Acta Oncol.* **34**, 727–734 (1995).
64. Y. Imamura and S. Mizuno, Mortality trends of rectal cancer in Japan: 1960–2000. *Jpn. J. Clin. Oncol.* **34**, 107–108 (2004).
65. M. D. Levitt, D. M. Millar and J. O. Stewart, Rectal cancer after pelvic irradiation. *J. R. Soc. Med.* **83**, 152–154 (1990).
66. H. Nakatsuka, Y. Shimizu, T. Yamamoto, I. Sekine, H. Ezaki, E. Tahara, M. Takahashi, T. Shimoyama, N. Mochinaga and M. Tomita, Colorectal cancer incidence among atomic bomb survivors, 1950–80. *J. Radiat. Res. (Tokyo)* **33**, 342–361 (1992).
67. D. M. Parkin, F. Bray, J. Ferlay and P. Pisani, Global cancer statistics, 2002. *CA Cancer J. Clin.* **55**, 74–108 (2005).
68. H. Yoshizawa, Hepatocellular carcinoma associated with hepatitis C virus infection in Japan: Projection to other countries in the foreseeable future. *Oncology* **62** (Suppl. 1), 8–17 (2002).
69. H. Tanaka, T. Hiyama, H. Tsukuma, Y. Okubo, H. Yamano, A. Kitada and I. Fujimoto, Prevalence of second generation antibody to hepatitis C virus among voluntary blood donors in Osaka, Japan. *Cancer Causes Control* **5**, 409–413 (1994).
70. K. Tanaka, T. Hirohata, K. Fukuda, A. Shibata, H. Tsukuma and T. Hiyama, Risk factors for hepatocellular carcinoma among Japanese women. *Cancer Causes Control* **6**, 91–98 (1995).
71. G. B. Sharp, T. Mizuno, J. B. Cologne, T. Fukuhara, S. Fujiwara, S. Tokuoka and K. Mabuchi, Hepatocellular carcinoma among atomic bomb survivors: Significant interaction of radiation with hepatitis C virus infections. *Int. J. Cancer* **103**, 531–537 (2003).
72. G. Randi, A. Altieri, S. Gallus, S. Franceschi, E. Negri, R. Talamini and C. La Vecchia, History of cirrhosis and risk of digestive tract neoplasms. *Ann. Oncol.* **16**, 1551–1555 (2005).
73. M. Pandey, Risk factors for gallbladder cancer: A reappraisal. *Eur. J. Cancer Prev.* **12**, 15–24 (2003).
74. S. Misra, A. Chaturvedi, N. C. Misra and I. D. Sharma, Carcinoma of the gallbladder. *Lancet Oncol.* **4**, 167–176 (2003).
75. T. Matsuba, D. Qui and M. Kurosawa, Overview of epidemiology of bile duct and gallbladder cancer focusing on the JACC Study Group. *J. Epidemiol.* **2 S1** (Suppl.), 150–160 (2005).
76. L. B. Travis, M. Hauptmann, L. K. Gaul, H. H. Storm, M. B. Goldman, U. Nyberg, E. Berger, M. L. Janower, P. Hall and M. Andersson, Site-specific cancer incidence and mortality after cerebral angiography with radioactive Thorotrast. *Radiat. Res.* **160**, 691–706 (2003).
77. IARC, *Tobacco Smoke and Involuntary Smoking. IARC Monographs on the Evaluation of Carciongenic Risks to Humans,* Vol. 83. International Agency for Research on Cancer, Lyon, 2004.
78. K. E. Andersen, J. D. Potter and T. M. Mack, Pancreatic cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 725–771. Oxford University Press, New York, 1996.
79. L. B. Travis, R. E. Curtis, H. Storm, P. Hall, E. Holowaty, F. E. Van Leeuwen, B. A. Kohler, E. Pukkala, C. F. Lynch and J. D. Boice, Jr., Risk of second malignant neoplasms among long-term survivors of testicular cancer. *J. Natl. Cancer Inst.* **89**, 1429–1439 (1997).
80. Y. Imamura and T. Sobue, Cancer statistics digest. Mortality trend of colon, rectal, liver, "gallbladder and biliary tract" and pancreas cancer in Japan by birth cohort. *Jpn. J. Clin. Oncol.* **34**, 491–493 (2004).
81. S. D. Stellman, T. Takezaki, L. Wang, Y. Chen, M. L. Citron, M. V. Djordjevic, S. Harlap, J. E. Muscat, A. I. Neugut and K. Aoki, Smoking and lung cancer risk in American and Japanese men: An international case-control study. *Cancer Epidemiol. Biomarkers Prev.* **10**, 1193–1199 (2001).
82. S. Kaneko, K. B. Ishikawa, I. Yoshimi, T. Marugame, C. Hamashima, K. Kamo, S. Mizuno and T. Sobue, Projection of lung cancer mortality in Japan. *Cancer Sci.* **94**, 919–923 (2003).
83. G. Pershagen, G. Akerblom, O. Axelson, B. Clavensjo, L. Damber, G. Desai, A. Enflo, F. Lagarde, H. Mellander and M. Svartengren, Residential radon exposure and lung cancer in Sweden. *N. Engl. J. Med.* **330**, 159–164 (1994).
84. E. S. Gilbert, M. Stovall, M. Gospodarowicz, F. E. Van Leeuwen, M. Andersson, B. Glimelius, T. Joensuu, C. F. Lynch, R. E. Curtis and L. B. Travis, Lung cancer after treatment for Hodgkin's disease: focus on radiation effects. *Radiat. Res.* **159**, 161–173 (2003).
85. M. Ichihashi, K. Naruse, S. Harada, T. Nagano, T. Nakamura, T. Suzuki, N. Wadabayashi and S. Watanabe, Trends in non-melanoma skin cancer in Japan. *Recent Results Cancer Res.* **139**, 263–273 (1995).
86. R. E. Shore, Occupational radiation studies: Status, problems, and prospects. *Health Phys.* **59**, 63–68 (1990).
87. M. R. Karagas, J. A. McDonald, E. R. Greenberg, T. A. Stukel, J. E. Weiss, J. A. Baron and M. M. Stevens, Risk of basal cell and squamous cell skin cancers after ionizing radiation therapy. For The Skin Cancer Prevention Study Group. *J. Natl. Cancer Inst.* **88**, 1848–1853 (1996).
88. E. Ron, B. Modan, D. Preston, E. Alfandary, M. Stovall and J. D. Boice, Jr., Radiation-induced skin carcinomas of the head and neck. *Radiat. Res.* **125**, 318–325 (1991).
89. J. D. Boice, Jr., D. Preston, F. G. Davis and R. R. Monson, Frequent chest X-ray fluoroscopy and breast cancer incidence among tuberculosis patients in Massachusetts. *Radiat. Res.* **125**, 214–222 (1991).

90. G. R. Howe and J. McLaughlin, Breast cancer mortality between 1950 and 1987 after exposure to fractionated moderate-dose-rate ionizing radiation in the Canadian fluoroscopy cohort study and a comparison with breast cancer mortality in the atomic bomb survivors study. *Radiat. Res.* **145**, 694–707 (1996).

91. R. E. Shore, N. Hildreth, E. Woodard, P. Dvoretsky, L. Hempelmann and B. Pasternack, Breast cancer among women given X-ray therapy for acute postpartum mastitis. *J. Natl. Cancer Inst.* **77**, 689–696 (1986).

92. N. G. Hildreth, R. E. Shore and P. M. Dvoretsky, The risk of breast cancer after irradiation of the thymus in infancy. *N. Engl. J. Med.* **321**, 1281–1284 (1989).

93. A. Mattsson, B. I. Ruden, P. Hall, N. Wilking and L. E. Rutqvist, Radiation-induced breast cancer: Long-term follow-up of radiation therapy for benign breast disease. *J. Natl. Cancer Inst.* **85**, 1679–1685 (1993).

94. M. Lundell, A. Mattsson, P. Karlsson, E. Holmberg, A. Gustafsson and L. E. Holm, Breast cancer risk after radiotherapy in infancy: A pooled analysis of two Swedish cohorts of 17,202 infants. *Radiat. Res.* **151**, 626–632 (1999).

95. C. M. Ronckers, C. A. Erdmann and C. E. Land, Radiation and breast cancer: A review of current evidence. *Breast Cancer Res.* **7**, 21–32 (2005).

96. M. Tokunaga, C. E. Land, S. Tokuoka, I. Nishimori, M. Soda and S. Akiba, Incidence of female breast cancer among atomic bomb survivors, 1950–1985. *Radiat. Res.* **138**, 209–223 (1994).

97. D. Grady and V. L. Ernster, Endometrial cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 1058–1089. Oxford University Press, New York, 1996.

98. M. Schiffman and P. E. Castle, Human papillomavirus: Epidemiology and public health. *Arch. Pathol. Lab. Med.* **127**, 930–934 (2003).

99. A. Ioka, H. Tsukuma, W. Ajiki and A. Oshima, Trends in uterine cancer incidence in Japan 1975–98. *Jpn. J. Clin. Oncol.* **33**, 645–646 (2003).

100. P. G. Smith and R. Doll, Late effects of x irradiation in patients treated for metropathia haemorrhagica. *Br. J. Radiol.* **49**, 224–232 (1976).

101. M. Ewertz and O. M. Jensen, Trends in the incidence of cancer of the corpus uteri in Denmark, 1943–1980. *Am. J. Epidemiol.* **119**, 725–732 (1984).

102. W. D. Atkinson, D. V. Law, K. J. Bromley and H. M. Inskip, Mortality of employees of the United Kingdom Atomic Energy Authority, 1946–97. *Occup. Environ. Med.* **61**, 577–585 (2004).

103. A. J. Sigurdson, M. M. Doody, R. S. Rao, D. M. Freedman, B. H. Alexander, M. Hauptmann, A. K. Mohan, S. Yoshinaga, D. A. Hill and M. S. Linet, Cancer incidence in the US radiologic technologists health study, 1983–1998. *Cancer* **97**, 3080–3089 (2003).

104. F. L. Wong, M. Yamada, H. Sasaki, K. Kodama, S. Akiba, K. Shimaoka and Y. Hosoda, Noncancer disease incidence in the atomic-bomb survivors: 1958–1986. *Radiat. Res.* **135**, 418–430 (1993).

105. M. Yamada, F. L. Wong, S. Fujiwara, M. Akahoshi and G. Suzuki, Noncancer disease incidence in atomic bomb survivors, 1958–1998. *Radiat. Res.* **161**, 622–632 (2004).

106. S. Kawamura, F. Kasagi, K. Kodama, S. Fujiwara, M. Yamada, K. Ohama and K. Oto, Prevalence of uterine myoma detected by ultrasound examination in the atomic bomb survivors. *Radiat. Res.* **147**, 753–758 (1997).

107. C. La Vecchia, Epidemiology of ovarian cancer: A summary review. *Eur. J. Cancer Prev.* **10**, 125–129 (2001).

108. K. Tamakoshi, T. Kondo, H. Yatsuya, Y. Hori, F. Kikawa and H. Toyoshima, Trends in mortality (1950–1977) and incidence (1975–1993) of malignant ovarian neoplasm among Japanese women: analysis by age, time, and birth cohort. *Gynecol. Oncol.* **83**, 64–71 (2001).

109. A. Ioka, H. Tsukuma, W. Ajiki and A. Oshima, Ovarian cancer incidence and survival by histologic type in Osaka, Japan. *Cancer Sci.* **94**, 292–296 (2003).

110. J. K. Wagoner, Leukemia and other malignancies following radiation therapy for gynecological disorders. In *Radiation Carcinogenesis: Epidemiology and Biological Significance* (J. D. Boice, Jr. and J. F. Fraumeni, Jr., Eds.). Raven Press, New York, 1984.

111. S. C. Darby, G. Reeves, T. Key, R. Doll and M. Stovall, Mortality in a cohort of women given X-ray therapy for metropathia haemorrhagica. *Int. J. Cancer* **56**, 793–801 (1994).

112. H. A. Weiss, S. C. Darby and R. Doll, Cancer mortality following X-ray treatment for ankylosing spondylitis. *Int. J. Cancer* **59**, 327–338 (1994).

113. S. Tokuoka, K. Kawai, Y. Shimizu, K. Inai, K. Ohe, T. Fujikura and H. Kato, Malignant and benign ovarian neoplasms among atomic bomb survivors, Hiroshima and Nagasaki, 1950–80. *J. Natl. Cancer Inst.* **79**, 47–57 (1987).

114. R. K. Ross and D. Schottenfeld, Prostate cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 1180–1206. Oxford University Press, New York, 1996.

115. J. K. McLaughlin, W. J. Blot, S. S. Devesa and J. F. Fraumeni, Jr., Renal cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 1142–1155. Oxford University Press, New York, 1996.

116. B. J. Drucker, Renal cell carcinoma: current status and future prospects. *Cancer Treat. Rev.* **31**, 536–545 (2005).

117. D. T. Silverman, A. S. Morrison and S. S. Devesa, Bladder cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 1156–1179. Oxford University Press, New York, 1996.

118. P. D. Inskip, M. S. Linet and E. F. Heineman, Etiology of brain tumors in adults. *Epidemiol. Rev.* **17**, 382–414 (1995).

119. E. Ron, B. Modan, J. D. Boice, Jr., E. Alfandary, M. Stovall, A. Chetrit and L. Katz, Tumors of the brain and nervous system after radiotherapy in childhood. *N. Engl. J. Med.* **319**, 1033–1039 (1988).

120. P. Karlsson, E. Holmberg, M. Lundell, A. Mattsson, L. E. Holm and A. Wallgren, Intracranial tumors after exposure to ionizing radiation during infancy: a pooled analysis of two Swedish cohorts of 28,008 infants with skin hemangioma. *Radiat. Res.* **150**, 357–364 (1998).

121. M. P. Little, F. de Vathaire, A. Shamsaldin, O. Oberlin, S. Campbell, E. Grimaud, J. Chavaudra, R. G. Haylock and C. R. Muirhead, Risks of brain tumour following treatment for cancer in childhood: modification by genetic factors, radiotherapy and chemotherapy. *Int. J. Cancer* **78**, 269–275 (1998).

122. E. Ron, Thyroid cancer. In *Cancer Epidemiology and Prevention* (D. Schottenfeld and J. F. Fraumeni, Jr., Eds.), pp. 1000–1021. Oxford University Press, New York, 1996.

123. J. W. Wood, H. Tamagaki, S. Neriishi, T. Sato, W. F. Sheldon, P. G. Archer, H. B. Hamilton and K. G. Johnson, Thyroid carcinoma in atomic bomb survivors Hiroshima and Nagasaki. *Am. J. Epidemiol.* **89**, 4–14 (1969).

124. S. Akiba, J. Lubin, E. Ezaki, E. Ron, T. Ishimaru, M. Asano, Y. Shimizu and H. Kato, *Thyroid Cancer Incidence among Atomic Bomb Survivors, Hiroshima and Nagasaki, 1958–1979.* Radiation Effects Research Foundation, Hiroshima, 1991.

125. E. Ron, J. H. Lubin, R. E. Shore, K. Mabuchi, B. Modan, L. M. Pottern, A. B. Schneider, M. A. Tucker and J. D. Boice, Jr., Thyroid cancer after exposure to external radiation: A pooled analysis of seven studies. *Radiat. Res.* **141**, 259–277 (1995).

126. United Nations Scientific Committee on the Effects of Atomic Radiation, *Sources, Effects, and Risks of Ionizing Radiation, with Annexes, Volume II: Effects.* United Nations, New York, 2000.

127. A. Virtanen, E. Pukkala and A. Auvinen, Incidence of bone and soft tissue sarcoma after radiotherapy: A cohort study of 295,712 Finnish cancer patients. *Int. J. Cancer* **118**, 1017–1021 (2006).

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DNA damage induced ubiquitylation of RFC2 subunit of RFC complex

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Running title: RPA-sensitive ubiquitylation of RFC2

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Many proteins involved in DNA replication and repair undergo post-translational modifications such as phosphorylation and ubiquitylation. PCNA (Proliferating Cell Nuclear Antigen, a homo-trimeric protein that encircles double-stranded DNA to function as a sliding clamp for DNA polymerases) is mono-ubiquitylated by the RAD6-RAD18 complex, and further poly-ubiquitylated by the RAD5-MMS2-UBC13 complex, in response to various DNA-damaging agents. PCNA mono- and poly-ubiquitylation activate an error-prone translesion synthesis pathway and an error-free pathway of damage avoidance, respectively. Here, we show that RFC (Replication Factor C, a hetero-pentameric protein complex that loads PCNA onto DNA) is also ubiquitylated in a RAD18-dependent manner in cells treated with alkylating agents or H_2O_2. A mutant form of RFC2 with a D228A substitution (corresponding to a yeast Rfc4 mutation that reduces an interaction with RPA, a

ssDNA-binding protein) is heavily ubiquitylated in cells, even in the absence of DNA damage. Furthermore, RFC2 was ubiquitylated by the RAD6-RAD18 complex *in vitro* and its modification was inhibited in the presence of RPA. The inhibitory effect of RPA on RFC2 ubiquitylation was relatively specific since RAD6-RAD18-mediated ubiquitylation of PCNA was RPA-insensitive. Our findings suggest that RPA plays a regulatory role in DNA damage responses via repression of RFC2 ubiquitylation in human cells.

Cellular DNA is continuously damaged by a vast variety of endogenous and exogenous genotoxicants. When genomic DNA is damaged, cells respond by activation of complex signaling network that delay cell-cycle progression, induce repair of lesions, activate damage tolerance pathways and trigger apoptosis or senescence (1,2). It is hypothesized that DNA damage-inducible signaling pathways serve important tumor-suppressive roles and prevent mutations that could lead to malignancy. Various genotoxins elicit different forms of DNA damage and result in distinct signal transduction pathways and biological outcomes. Distal steps of DNA damage-induced checkpoint signaling pathways that result in inhibition of the cell cycle are relatively well understood (3,4). However, molecular details of proximal signaling events and lesion-specific DNA damage recognition events are less clear.

DNA replication and repair requires the coordinated actions of multiple proteins on small regions of DNA. A limited number of proteins serve to coordinate multiple replication and repair events. Some proteins function commonly in DNA replication and repair, and frequently have a crucial role in both processes. Three such examples are replication protein A (RPA), proliferating cell nuclear antigen (PCNA) and replication factor C (RFC). RPA was originally identified as a eukaryotic single-stranded DNA binding protein essential for *in vitro* replication of SV40 DNA (5,6). PCNA is a trimer of three identical subunits arranged head-to-tail to generate a ring-like structure with a large central cavity for encircling DNA. It is well established that PCNA provides a mobile platform to serve as anchor and processivity factor for DNA polymerases during chromosomal replication (7,8). PCNA is loaded onto the primer-template junction in an ATP-dependent manner by a multi-protein clamp loader, RFC (9,10). RFC binds preferentially to double-stranded/single stranded junctions with a recessed 3' end, which is the DNA target for PCNA loading.

RPA, PCNA and RFC are key proteins that play central roles in DNA replication, participating in competitive polymerase switching during lagging strand synthesis. The DNA polymerase -primase complex (Pol) that synthesizes an RNA-DNA hybrid primer

requires contact with RPA to remain stably attached to the primed site. For processive DNA synthesis to follow, Pol must be replaced by DNA polymerase (Pol). Replacement of Pol by Pol is initiated by interactions between RFC and RPA, which disrupt Pol -RPA interactions and result in removal of Pol from DNA. After RFC loads PCNA onto the primed site, Pol associates with PCNA by displacing RFC. The switching process is indeed coordinated by RPA, via cooperative interactions with PCNA and RFC (11,12). RPA, RFC, and PCNA also play key roles in DNA repair by interacting with many DNA repair enzymes (13-15). Such interactions are believed to play roles in DNA damage recognition and in recruiting and positioning of DNA repair enzymes.

RFC consists of five different subunits, which are homologous to one another and are members of the AAA+ family of ATPases (16,17). The RFC1(p140) subunit is sometimes referred to as 'large subunit', as it contains both N- and C-terminal extensions beyond its region of homology with the four 'small' subunits. The four small RFC subunits are designated RFC2(p40), RFC3(p36), RFC4(p37) and RFC5(p38) in mammals. Three protein complexes with resemblance to RFC have been recently described, which are involved in maintaining genome stability. These RFC-like complexes (RLCs) share four common small subunits (RFC2-5) and each carry a unique large subunit (RAD17, CTF18 or ELG1) replacing the RFC1. These RLCs are involved in the checkpoint response (RAD17-RFC), sister chromatid cohesion (CTF18-RFC) and maintenance of genome stability (ELG1-RFC) (18,19).

DNA-damage sensors and repair proteins must react in a rapid and efficient manner to execute their functions. Frequently, the regulation of these proteins involves post-translational modifications, such as phosphorylation and ubiquitylation, to help modulate the assembly and disassembly of complexes, and to assist targeting and the regulation of enzymatic activity in a timely manner. For example, RPA is hyper-phosphorylated upon DNA damage or replication stress by several checkpoint kinases (20). Hyperphosphorylation alters RPA-DNA and RPA-protein interactions (15,21). Recent studies in the DNA-repair field have highlighted the expanding role of ubiquitylation in the regulation of diverse DNA-repair processes and pathways. One of the most striking examples of how ubiquitylation can affect protein function is that of PCNA in budding yeast *Saccharomyces cerevisiae*. Following DNA damage, PCNA can be mono-ubiquitylated, or poly-ubiquitylated on the K164 residue and each modification results in a different outcome with respect to DNA synthesis and repair (22,23). Mono-ubiquitylated PCNA directs translesion synthesis (TLS) via error-prone DNA

polymerases, while poly-ubiquitylated PCNA is associated with an error-free DNA repair pathway (22,23). Mammalian PCNA also undergoes mono-ubiquitylation after UV irradiation, and mono-ubiquitylated PCNA preferentially binds to TLS polymerases that contain one or two copies of ubiquitin-binding domains (24-27).

In contrast to RPA and PCNA, damage-dependent modification of RFC has not been described. Recent studies have significantly broadened the scope of the role of ubiquitylation to include regulatory functions in DNA repair and damage response pathways. Therefore, in this report we investigated whether the clamp loader RFC is likewise subjected to regulated modification. We have examined the modification of all subunits in RFC and RLCs. We demonstrate that RFC2 and RFC4 are ubiquitylated following treatment of cells with alkylating agents. The ubiquitylation is partially dependent on RAD18. Surprisingly, RPA inhibits the RAD18-dependent ubiquitylation of RFC2. Our results suggest that RFC regulates the DNA damage response pathway via interaction with RPA and ubiquitylation.

Experimental Procedures

Plasmid constructs- To generate pCDNA.RFC2(p40)Flag and pCDNA.RFC2(p40)HA, human p40 coding region was amplified by PCR as a EcoRI-XhoI fragment. The PCR product was inserted into the EcoRI-XhoI site either of pCDNA-C-Flag or pCDNA-C-HA. To generate pCAGGS.RFC2(p40), the human p40 coding region was amplified by PCR as a SalI-XhoI fragment. The PCR product was inserted into the XhoI site of pCAGGS. pCDNA-C-Flag and pCDNA-C-HA was constructed by inserting the Flag or HA epitope into the XhoI-XbaI site of pCDNA3.1. Expression plasmids containing human RFC1-FLAG, human FLAG-RAD17, human FLAG-CTF18, human FLAG-p38, human FLAG-p37, human FLAG-p36 were constructed by inserting their cDNA described in (28) into pCDNA3. Although N-terminal and C-terminal tagged forms of each RFC2 subunit were used, the presence of the epitope-tag did not affect RFC2 regulation at least in the context of experiments reported in this study. pCAGGS.Flag-Ubiquitin and pCAGGS.hRAD18 were constructed as previously described (25). The expression plasmids for human RFC, PCNA were described earlier (29,30) and that for human RPA, p11d-tRPA (31), was a generous gift of Dr. Marc S. Wold (University of Iowa College of Medicine, Iowa City, Iowa). Mouse E1 expression vector RLC(32,33) was a generous gift of Dr. Hideyo Yasuda (School of Life Science, Tokyo University of Pharmacy, and Life Science, Tokyo, Japan). Human cDNAs for RAD6A and RAD18 amplified from a HeLa cDNA library by PCR introducing a NdeI site at

the start codon were cloned together into pET20b(+) (Novagen) as an artificial operon. After cloning the PCR fragments, the nucleotide sequences were verified. All the expression plasmids of PCNA, RPA, RFC, E1, RAD6A and RAD18 were designed for production of intact proteins, without any affinity tags.

Cell culture and transfection- 293A and HCT116 cells were grown in DMEM supplemented with 10% fetal bovine serum (FBS). HCT116 *RAD18*$^{-/-}$ cells were established as described previously (25). Cells were transfected with Lipofectamine Plus (Invitrogen) or Lipofectamine 2000 according to the manufacture's protocol. 2.4 μg of plasmid DNA was used to transfect each 6 cm plate of cells. Transfected cells were treated with genotoxins 24 hr post-transfection.

Genotoxin and Inhibitor Treatments- Asynchronous cell cultures were grown to ca. 80% confluency. For UV treatment, cells were washed with PBS, exposed to UV light (254 nm) at a fluence rate of 43 Jm^{-2}/s. For genotoxin and inhibitor treatment, hydroxyurea (HU, 1 M in H_2O), aphidicolin (dissolved in DMSO), methyl methanesulfonate (MMS, dissolved in DMSO), ethyl methanesulfonate (EMS, dissolved in DMSO), N-methyl-N'-nitro-N-nitrosoguanidine (MNNG, dissolved in DMSO), H_2O_2 (diluted in PBS), mitomycin C (MMC), bleomycin (Bleo, dissolved in H_2O) or camptothecin (CPT, dissolved in DMSO), was added to the culture media to give a final concentration of 2 mM, 0.025mM, 0.1-1.7 mM, 20 mM, 0.7 mM, 0.5 mM, 0.01mM, 0.05mg/ml or 20 nM, respectively and cells exposed for 8 hr unless otherwise stated.

Antibodies- A mouse monoclonal antibody against *Drosophila* RFC40 (anti-dRFC40) was used for probing human RFC2(p40). A hybridoma cell line producing anti-dRFC40 antibody is a kind gift from Dr. Gerald M. Rubin (University of California, Berkeley) and monoclonal antibody was purified as described previously (34). To test whether anti-dRFC40 antibody cross-reacts with human RFC2(p40), an HA epitope-tagged form of hRFC2(p40) was over-expressed in 293A cells by transfection, cell lysate was recovered 24 hr post-transfection, and then immunoblotted with either anti-dRFC40 or anti-HA antibody. An anti-dRFC40-reactive protein band migrating at 40 kDa was clearly observed only in extracts from HA-hRFC2(p40)-transfected cells and corresponded to the species detected with an anti-HA antibody (Supplementary Fig. 1). Therefore, the anti dRFC40 antibody recognizes human RFC2(p40). To avoid confusion we refer to the anti-dRFC40 antibody as 'anti-RFC2' antibody in this report.

Other commercial antibodies used in this study are: anti-HA (Y-11, Santa Cruz Biotechnology), anti-FLAG (M2, Sigma), anti-RFC1 (H-300, Santa Cruz Biotechnology), anti-RAD17 (H-3, Santa Cruz Biotechnology),

anti-Tubulin (B-5-1-2, Sigma), anti-Histone H3 (6.6.2, Upstate and ab1791, Abcam) and anti-PCNA (PC10, Oncogene).

Preparation of Cell Lysate and Chromatin Fraction- 293A cells in a 3.5 cm or 6 cm dish were washed twice with ice-cold PBS and then harvested into RIPA buffer (1xPBS, 1%NP-40, 0.5% sodium deoxycholate, 0.1%SDS, 1mM PMSF, 1mM sodium orthovanadate, and protease inhibitor (Nacalai)). The cell suspensions were incubated for 30 min on ice, and then the NP40-0.1%SDS-insoluble fraction and soluble fraction were separated by centrifugation. The soluble fraction was used as Supernatant (Sup) fraction. The resultant pellet was washed with PIPA buffer four times and then sonicated after adding SDS-PAGE loading buffer (7% Glycerol, 22% SDS, 50 mM Tris-HCl (pH6.8), 5% b-mercaptoethanol). The resultant solution was used as Chromatin fraction. We have confirmed few contaminations in each Sup and Chromatin fraction using anti-Tubulin and anti-Histone H3 antibodies (Supplementary Fig. 2).

SDS-PAGE and Western blotting- Cell extracts were resolved by electrophoresis on 7.5 or 10% SDS-PAGE gels. Following transfer onto PVDF or nitrocellulose filters, the blots were incubated with antibodies and immunoblots were visualized by enhanced chemiluminescence (ECL, Amersham Pharmacia or DURA, Pierce), according to the manufacture's instructions.

Immunoprecipitation- Cell extracts were incubated with monoclonal mouse anti RFC2 (dRFC4(p40)) antibody for 1 hr at 4 °C, and then with 25 μl of A/G-agarose (Santa Cruz). After incubation for overnight at 4 °C, the beads were washed with PBS three times, boiled in Laemmli buffer for 5 min, and the bound proteins were analyzed by electrophoresis and immunoblotting.

Protein Purification- Human RFC, PCNA, RPA were purified as described (29,30). Mouse E1 was overproduced in insect cells and purified as described (35). Human RAD6A-RAD18 complex was overproduced in *E. coli* cells and then purified by column chromatography (phosphocellulose, heparin sepharose, MonoQ and gel filtration) from *E. coli* cell lysate. Protein concentrations were determined by Bio-Rad protein assay kit (Bio-Rad) using BSA as the standard. Bovine ubiquitin was purchased from Sigma.

in vitro ubiquitylation assay- The reaction mixture (25 μl) contained 20 mM HEPES-NaOH (pH 7.5), 50 mM NaCl, 0.2 mg/ml BSA, 1 mM DTT, 10 mM MgCl2, 1 mM ATP, 33 fmol of singly primed single stranded M13 mp18 DNA (30) , 1.0 μg (9.1 pmol) of RPA, 86 ng (1.0 pmol as a trimer) of PCNA, 75 ng (260 fmol) of RFC, and 100 ng (850 fmol) of mouse E1, 175 ng (2.4 pmol) of RAD6A-RAD18 complex, and 12.5 μg (1460 pmol) of ubiquitin. After incubation at 30°C for 60 min, reactions were terminated with 2microl

of 300 mM EDTA.

Structural model building- Homology modeling of the human clamp-loader/clamp complex was performed using MODELLER v7.7 (36). The homologous structures were defined using the fold recognition server FORTE (37). The atomic coordinate of the clamp/clamp-loader complex (PDB:1SXJ) was selected as a templates for model building. Before submission to MODELLER, the sequence–structure alignment obtained from FORTE was used. Due to the lack of the template structure, the N-terminal 582 residues of human RFC1 were not modeled. The figures were prepared using MOLMOL (38). Coloring of each RFC subunits and PCNA was according to Figure 2 in the review (39).

RESULTS

Specific DNA damaging agents induce modification of RFC2. To analyze the modification of each subunit of the RFC complex, a Flag epitope-tagged form of each subunit of RFC and RLCs was expressed in human 293A cells. Transfected cells were treated with UV, -ray, hydroxyurea (HU) or MMS, and then cell extracts were prepared. The cell extracts were separated into NP40-insoluble chromatin fractions (CF) and soluble fractions (Sup). RFC and RLC subunits in each fraction were analyzed by SDS-PAGE and Western blotting (Fig. 1A). Following MMS treatment all of the subunits, except for CTF18 and RFC5, accumulated in the chromatin fraction, whereas no accumulation was observed following treatments with UV, -ray or HU. Levels of soluble CTF18 and RFC5 decreased after MMS treatment, although we did not detect concomitant increases in the chromatin-bound levels of these subunits (Fig. 1A). Taken together, the results of Fig. 1A demonstrate that the levels and subcellular distribution of RFC and RLC subunits are regulated in response to MMS.

It was important to determine whether endogenous RFC and RLC subunits were also redistributed to chromatin in response to MMS. Therefore, we determined the effects of MMS on endogenous RFC1, RAD17 or RFC2 proteins for which good antibodies are available. As shown in Fig. 1B, endogenous RFC1, RAD17 and RFC2 accumulated in the chromatin fraction of MMS-treated 293A cells. Similar to ectopically expressed tagged proteins, endogenous RFC subunits are redistributed to chromatin in response to MMS treatment.

Interestingly, we observed prominent forms of ectopically-expressed RFC2 and RFC4 that migrated with reduced electrophoretic mobility on SDS-PAGE gels in chromatin fractions from MMS-treated 293A cells (Fig. 1A, lane 7). Electrophoretically-retarded species of endogenous RFC2 were also evident in chromatin fractions of MMS-treated 293A cells (Fig. 1B, lane 7). The electrophoretically-shifted

form of RFC2 was more prominent than that of RFC4 (Fig. 1A). Therefore we focused on RFC2 and further analyzed its MMS-induced modification.

We performed quantitative analyses to determine the amount of chromatin-bound RFC2 relative to the soluble fraction in MMS-treated cells. In 293A cells ectopically expressing HA-tagged RFC2, more than 90% of the RFC2 accumulated to the chromatin fraction following 8 h of MMS treatment, whereas in untreated cells, less than 10% of RFC2 was present in the chromatin fraction (Supplementary Fig. 3). Following MMS treatment, we consistently detected two electrophoretically-retarded -RFC2-reactive proteins in the chromatin fraction. The apparent molecular mass of electrophoretically-retarded RFC2 is consistent with ubiquitylation. The two putative ubiquitylated forms of RFC2 (shown in Fig. 1) might correspond to species that are mono-ubiquitylated on different residues. However, we cannot exclude the possibility that modifications other than ubiquitin are also present on the shifted RFC2. Furthermore, smaller -RFC2-reactive proteins, possibly corresponding to degradation products, were detected in soluble and chromatin fractions from both control and MMS-treated cells (Fig. 1B and Supplementary Fig. 3).

The electrophoretically-retarded forms of RFC2 were induced by MMS in a dose-dependent manner (Fig. 1C). At lower concentrations of MMS (0.1 mM or 0.213 mM), no RFC2 band-shift was detectable. However, treatment with higher concentrations of MMS (0.425 mM, 0.85 mM or 1.7 mM) induced prominent electrophoretically-retarded forms of RFC2 on chromatin (Fig. 1C).

In the experiments described above, the cells were treated with MMS for 8 h. We subsequently examined the kinetics of RFC2 modification by treating 293A cells with MMS (0.85 mM) for 1 h and preparing samples for immunoblotting at 0, 2, 5 and 8 h following MMS-treatment. As shown in Fig. 1D, the shifted forms of RFC2 were detectable by 5 h after treatment of cells with MMS (lane 4). Similar to results of Fig. 1A, the genotoxin-induced RFC2 mobility shift was specific for MMS since UV irradiation (30 J/m^2; lanes 7-9) did not induce RFC modification at any time point tested (although as expected, UV induced PCNA mono-ubiquitylation under these experimental conditions). Conversely, little or no PCNA modification was detectable under the conditions used for the experiment shown in Fig. 1D (lanes 2-5), although low levels of PCNA ubiquitylation were observed when cells were treated with 0.85 mM of MMS for longer times (data not shown).

The results of Figs. 1A and 1D indicated that MMS-induced RFC2 modification is not a general response to DNA damage. To gain insight into the significance of RFC2 modification, 293A cells ectopically expressing