# Chapter 5

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## Detection of genotoxic and non-genotoxic carcinogens in *Xpc*$^{-/-}$*p53*$^{+/-}$ mice

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*"In for a round of overexposure, the thing mother nature provides"*

Bottle Up And Explode! - Elliott Smith, 1998

## Abstract

An accurate assessment of the carcinogenic potential of chemicals and pharmaceutical drugs is essential to protect humans and the environment. Therefore, substances are extensively tested before they are marketed to the public. Currently, the rodent two-year bioassay is used to assess the carcinogenic potential of substances. However, over the years it has become clear that this assay yields false positive results and also has several economic and ethical drawbacks including the use of large numbers of animals, the long duration, and the high cost. The need for a suitable alternative assay is therefore high. Previously, we have proposed the *Xpa*p53* mouse model as a very suitable alternative to the two-year bioassay. We now show the *Xpc*p53* mouse model preserves all the beneficial traits of the *Xpa*p53* model for short-term carcinogen identification and can identify both genotoxic and non-genotoxic carcinogens. Moreover, *Xpc*p53* mice appear to be more responsive than *Xpa*p53* mice towards several genotoxic and non-genotoxic carcinogens. Furthermore, *Xpc*p53* mice are far less sensitive than *Xpa*p53* mice for the toxic activity of DNA-damaging agents and as such clearly respond in a similar way as wild type mice do. The advantageous traits of the *Xpc*p53* model make it a better alternative for *in vivo* carcinogen testing than *Xpa*p53.* We propose that *Xpc*p53* mice are suited for routine short-term testing of both genotoxic and non-genotoxic carcinogens and as such are a suitable alternative to possibly replace the murine life time cancer bioassay.

## Introduction

Cancer ranks as one of the most frequent causes of death worldwide. Reasons for this high frequency in Western countries can mostly be attributed to lifestyle and environmental factors. Cancer incidence with a prominent hereditary cause is only ~5-10% [1]. Lifestyle and environmental factors are thought to enhance abnormalities in the (epi)genetic material of cells, thereby facilitating the onset of the disease [2]. Carcinogenesis is considered as a multi-step process. Although genomic instability is represented in virtually all tumors, it is believed to be an enabling characteristic that allows evolving populations of premalignant cells to reach the biological hallmarks that eventually characterizes them as cancerous [3;4]. In the current study we show that genomic instability could provide a suitable and alternative tool for prediction and risk assessment of carcinogens *in vivo*.

Cancer risk assessment follows a standard strategy, consisting of a qualitative (hazard identification) and a sub-sequential quantitative (dose-response analysis) component [5]. Several *in vitro* and *in vivo* genotoxicity assays are generally used for hazard identification of large numbers of chemicals for possible carcinogenic properties. A substance is suspected to be carcinogenic based on the results of *in vitro* and *in vivo* genotoxicity tests and a two-year bioassay is performed to obtain hazard confirmation and information with regards to cancer potency (*i.e.* dose-response analysis).

The current test strategy is not without disadvantages, given that genotoxicity tests are not perfect in predicting the carcinogenic potential of chemicals and are not designed to detect non-genotoxic carcinogens and hence can provide false negative results. Misclassification of these non-genotoxic carcinogens can have harmful effects on society and the environment. In addition, the two-year bioassay has several disadvantages. Firstly, the number of animals needed is large, plus the assay is highly time consuming and expensive. Moreover, there is considerable scientific doubt about the reliability of the assay, since too many false positive results have been observed. The need for a good alternative for genotoxic as well as non-genotoxic carcinogenicity testing is therefore high.

In previous studies we have demonstrated that the *Xpa*$^{-/-}$*Trp53*$^{+/-}$ mouse model (hereafter named *Xpa*p53*) is deficient in both global genome as well as transcription coupled nucleotide excision repair (GG-NER and TC-NER), and is heterozygous for p53 (*Trp53*$^{+/-}$), resulting in an increased cancer susceptibility upon carcinogen exposure as compared to wild type mice [6;7]. The *Xpa*p53* mice have a relatively low spontaneous tumor background and, when exposed to carcinogens, exhibit tumor types that are similar to those found in wild type (WT) C57BL/6J mice [6;7]. This increased susceptibility can be beneficial in carcinogenicity testing since the number of animals as well as the time of exposure can be decreased to accurately identify carcinogens. A concomitant advantage of the *Xpa*p53* model is its responsiveness to several non-genotoxic carcinogens [6]. A disadvantage of the *Xpa*p53* model is, however, its enhanced sensitivity towards toxicity, especially when induced by genotoxic carcinogens, making more quantitative (potency) comparisons unrelated to what is observed in wild type animals. Concentrations used for carcinogenicity testing in wild type mice cannot always be used in *Xpa*p53* mice, since these doses might be toxic ([8], unpublished results).

We and others have shown that, although *Xpa* and *Xpc*-deficient mice have many similarities in terms of their general cancer proneness, they exhibit a few striking differences. *Xpc* mice are less sensitive, as compared to *Xpa* mice, to the toxic effects of (mostly) genotoxic compounds [9;10]. It was suggested [9-12] that this difference in sensitivity is caused by the defect that *Xpa* mice have in the TC-NER

pathway. In this aspect *Xpa* mice resemble the NER-deficient *Csb* mice, which are also hypersensitive to genotoxicants (*e.g.* UV radiation) and have a defective TC-NER but a proficient GG-NER (this in contrast to *Xpa* mice). Given the fact that *Xpc* mice have an active TC-NER, and are therefore supposed to be less sensitive to genotoxic agents, we set out to test the use of the *Xpc*$^{-/-}$*Trp53*$^{+/-}$ mouse model (hereafter named *Xpc*p53)* for carcinogenicity testing. Above that, we tested whether *Xpc*p53* mice display the same response to non-genotoxic carcinogens as the *Xpa*p53* mouse model does.

## Material & Methods

### *Mice*

The generation of the NER-deficient *Xpc* and *Xpa* mouse models has been described previously [13;14]. *Xpc* and *Xpa* mice were crossed with *Trp53*$^{+/-}$ mice [15] to generate both *Xpc*p53* (used in this study for carcinogenicity studies and generation of primary hepatocytes) and *Xpa*p53* mice (used in this study to generate primary hepatocytes). All mouse models were generated in a pure C57BL/6J genetic background. Genotyping of the different mice was performed via allele-specific PCR analysis for altered genes as been described previously [9;15;16].

### *Chemicals*

2-Acetylaminofluorene (2-AAF, CAS #53-96-3), Diethylstilbestrol (DES, CAS #56-53-1), Di(2-ethylhexyl)phthalate (DEHP, CAS #117-81-7), Wyeth-14.643 (WY, CAS #50892-23-4), Phenacetin (Phe, CAS #62-44-2), Aflatoxin B1 (AFB1, CAS #1162-65-8) and Mitomycin C (MMC, CAS #50-07-7) were purchased from Sigma (St. Louis, MO, USA). Cyclosporin A (CsA, CAS #59865-13-3) was kindly provided by Novartis Pharma AG (Basel, Switzerland).

### *Experimental setup*

Short-term carcinogenicity studies were performed as follows: wild type and *Xpc*p53* mice (8-10 weeks old) were exposed through feed (Altromin, Lage, Germany) for 39 weeks to either a genotoxic carcinogen (GTXC), a non-genotoxic carcinogen (NGTXC) or control diet. After 39 weeks of exposure all animals were fed control diet for another 2 weeks. Both female and male mice (*n*=20 per exposure group, *n*=40 per untreated control group) were used. Table 1 gives an overview of the experimental setup of the (exposure) studies. The doses used were based on previous dose-range finding studies obtained with wild type or *Xpa* and *Xpa*p53* mice [6;17-19]. *Xpa*p53* data to compare with the studies in this manuscript were derived from previous short-term carcinogenicity studies [6;7].

The health status of the mice was monitored daily from weaning. Animals were weighed biweekly (Average body weight curves are depicted in Figure 1). Average weekly food uptake per cage was measured for the duration of the experiment for all cages. Weekly uptake, averaged per mice, is depicted in Supplemental Figure 1. During the entire duration of the experiment, animals were kept in the same stringently controlled (specific pathogen-free, spf) environment, fed *ad libitum*, and kept under a normal day/night rhythm (12hr/12hr). The microbiological status of the cohorts was monitored every 3 months. Animals were removed from the study when found moribund or dead.

Autopsy was performed on animals of all groups; several tissues were isolated from each animal and stored for further histopathology analysis (see below). The experimental setup of the studies was examined and approved by the institute's Ethical Committee on Animal Experimentation, in accordance with national and European legislation.

### *Pathology*

Based on previous *Xpa*p53* exposure studies, using the carcinogens as shown in Table 1, target tissues were selected based on the carcinogen in question and observed gross abnormalities during necropsy. Tissue samples (liver, kidney, spleen, urinary bladder, thymus, femur, pituitary, mesenteric lymph nodes, cervix and those tissues showing gross lesions) from each animal were preserved in a neutral aqueous phosphate-buffered 4% solution of formaldehyde solution. Tissues selected for detailed microscopic examination were processed, embedded in paraffin wax, sectioned at 4µm and stained with haematoxylin and eosin.

### *Isolation and culture of primary mouse hepatocytes*

Primary mouse hepatocytes were isolated from 8-10 weeks old male mice (C57/BL6, *Xpa*p53* and *Xpc*p53*) by a modified two-step collagenase perfusion technique (collagenase type IV, Sigma, St. Louis, MO, USA), as previously described [20]. Hepatocyte suspensions with at least 80% viability, determined by trypan blue exclusion, were seeded to 6-wells plates coated with 1 mg/ml neutralized collagen type I (BD Biosciences, Breda, The Netherlands) at 1.3 x $10^6$ cells per well. After two hours of incubation in a humidified atmosphere at 37˚C and 5% $CO_2$, unattached hepatocytes were removed by washing and a sandwich configuration was achieved by adding a second layer of neutralized collagen to the cells. After one hour, serum-free DMEM (Invitrogen, Bleiswijk, The Netherlands) was added, containing 2% penicillin/streptomycin (Invitrogen), 7 ng/ml glucagon (Sigma), 7.5 µg/ml hydrocortisone (Sigma) and 0.5 U/ml insulin (from bovine pancreas, Sigma). Cells were kept in serum-free medium and the culture medium was changed daily until exposures were performed.

### *Cytotoxicity analyses*

Cytotoxicity analyses were performed for Aflatoxin B1, Mitomycin C, Wyeth-14,643 and Cyclosporin A. Forty-six hours after isolation, hepatocytes were exposed for 24 hours to the substances dissolved in DMSO (AFB1, CsA and Wy) or PBS (MMC) at multiple doses (Figure 3). Final DMSO or PBS concentrations in medium were 0.5 % (v/v) in all exposure studies, including the vehicle controls. After 24 h cells were given fresh serum-free medium and after another 48 h (t = 72h) the cytotoxicity of all substances was tested, using the MTT reduction method [21], with some modifications. In short, cultures were incubated for one hour with 0.5 mg/ml MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Invitrogen). The medium was removed and the formazan crystals formed were solubilized in DMSO. Absorbance was measured in triplicate at 570 nm and a reference wavelength at 670 nm. Vehicle-treated cells were used as a solvent control and were taken as a 100% cell viability control. Dose-response calculations were done using PROAST software (www.rivm.nl/proast) [22].

### *Statistical analyses*

Statistical analyses on the (Kaplan-Meier plotted) survival curves were performed in PASW Statistics 17.0.2 (SPSS) using Log Rank (Mantel-Cox) analyses. Statistical analyses on the incidence of tumor bearing animals and preneoplastic lesions were performed using a one-sided Fisher's Exact test.

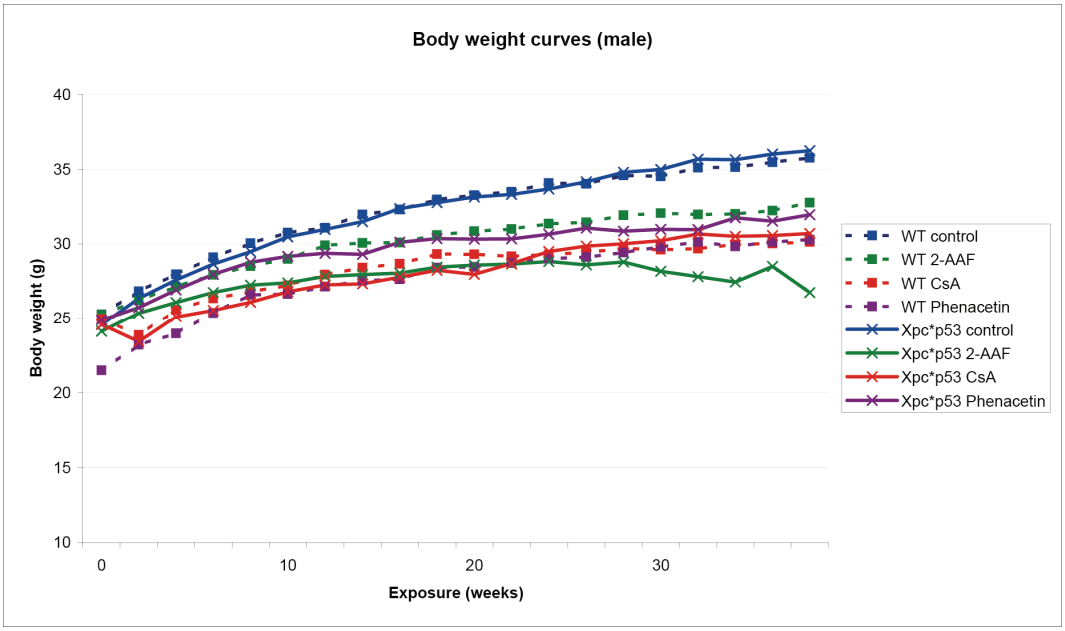
## Results

### *Divergent survival dynamics of wild type and Xpc*p53 mice after GTXC and NGTXC exposure*

Short-term (39 weeks) exposures studies with several human and rodent carcinogens (Table 1) were performed. We tested a range of different compounds, either with a genotoxic or non-genotoxic mode of action and with different carcinogenic potencies. In addition, untreated cohorts (male and female mice) were used as controls. Body weight dynamics of wild type and *Xpc*p53* mice were generally comparable (Figure 1). Exposure to WY induced a clear effect on body weight in both genotypes. All Wyeth-exposed mice (only females were used in this study) exhibited a body weight reduction from the start of exposure and maintained a very low body weight throughout the study. The decrease in body weight coincides with the decreased food uptake that was measured during the Wyeth exposure (Supplemental Figure 1). Exposure to 2-AAF resulted in lower body weights of *Xpc*p53* mice compared to wild type animals. This effect is accentuated near the end of the exposure due to intercurrent loss of mice (especially males). Food uptake of 2-AAF on average of the *Xpc*p53* male mice was a little lower than the wild type males, which could have contributed to the slight divergence in body weight between the genotypes (Supplemental information 1).

| Exposure | Dose | GTXC/NGTXC | Sex |
|---|---|---|---|
| Control | 0 | - | F + M |
| 2-Acetylaminofluorene (2-AAF) | 300 ppm | GTXC (human) | F + M |
| Phenacetin (Phe) | 7500 ppm | GTXC (human) | M |
| Cyclosporin A (CsA) | 500 ppm | NGTXC (human) | M |
| Diethylstilbestrol (DES) | 1.5 ppm | NGTXC (human) | F |
| Wyeth-14,643 (WY) | 250 ppm | NGTXC (rodent) | F |
| Di(2-ethylhexyl)phthalate (DEHP) | 6000 ppm | NGTXC (rodent) | F |

**Table 1.** Experimental setup. For all exposures both wild type and *Xpc*p53* mice were used.
GTXC = genotoxic carcinogen, NGTXC = non-genotoxic carcinogen



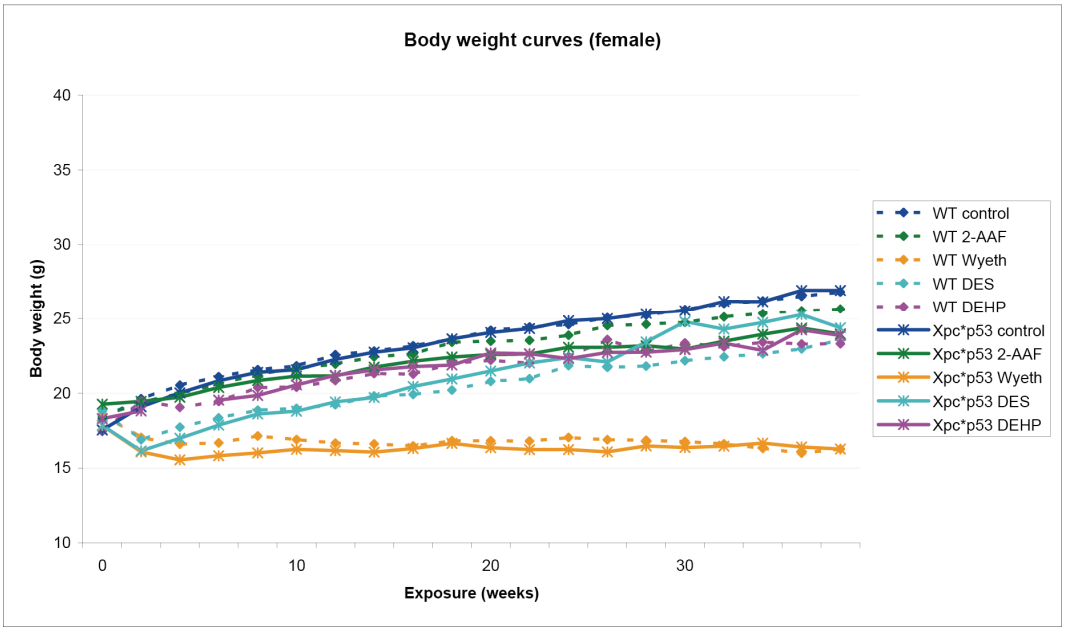
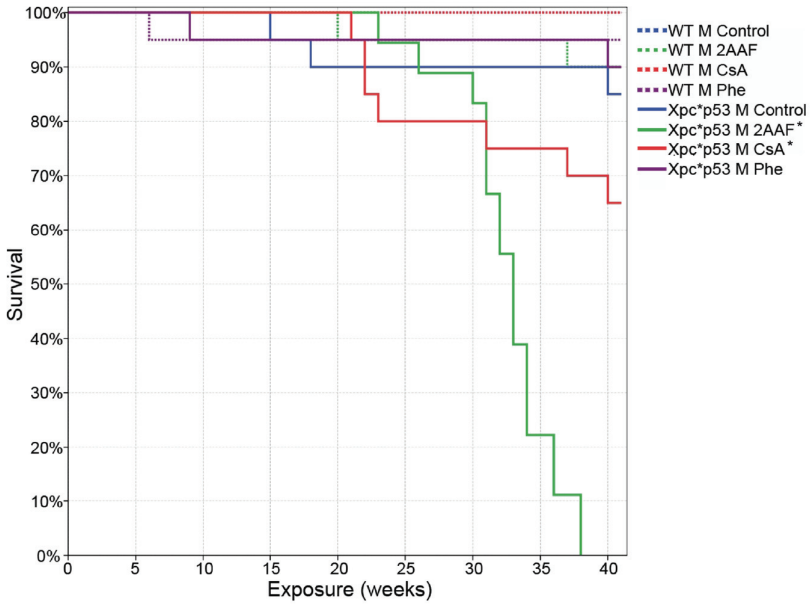




**Figure 1.** Body weight curves male (**A**) and female (**B**) mice. Body weight curves of wild type and *Xpc*p53* (male and female) mice during 39 week exposure to genotoxic or non-genotoxic carcinogens mixed through feed. Curves depict average absolute body weights.

A.




B.

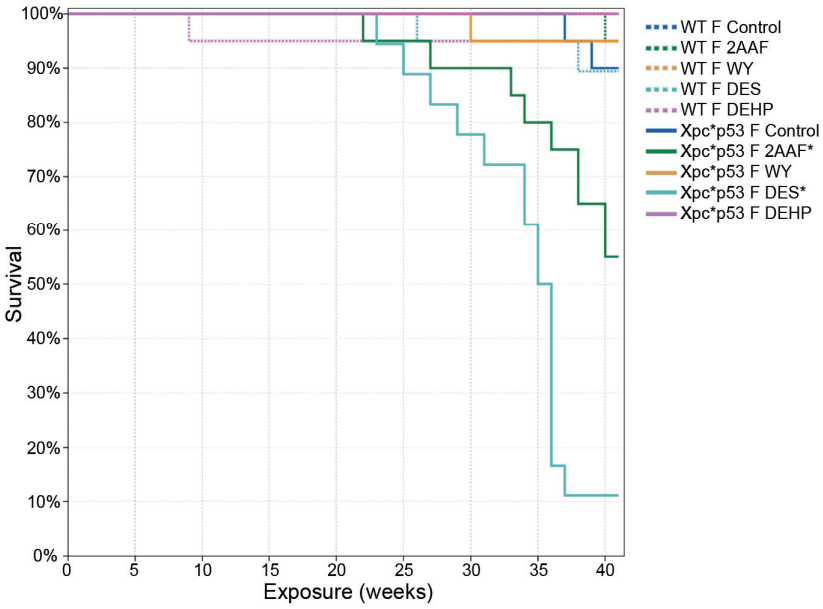



**Figure 2.** Survival curves of wild type and *Xpc*p53* male (**A**) and female (**B**) mice after short-term carcinogen exposure. Mice of 8-10 weeks old were exposed to genotoxic (2-AAF, Phe) and non-genotoxic (CsA, DES, WY, DEHP) carcinogens for 39 weeks, followed by a 2-week recovery period (see Table 1 for details).
* = $p<0.05$ compared to untreated group of same genotype.

The survival curves are depicted in Figure 2. Only a minor, non-significant ($p$<0.05) reduction in survival was observed in untreated *Xpc*p53* mice compared to the untreated wild type mice, indicating that wild type and *Xpc*p53* mice show similar spontaneous survival up to this age. Results demonstrated a clear and significant effect of exposure on survival of the *Xpc*p53* mice for the strong genotoxic agent 2-AAF ($p$<0.05); none of the male mice survived the 39 week duration of the exposure and had to be sacrificed intercurrently. Upon 2-AAF exposure, female *Xpc*p53* mice showed a less dramatic decrease in survival, but still 45% (9/20) did not survive the full 39 weeks of exposure ($p$<0.05). In contrast, only 10% (2/20, $p$>0.05) of the wild type male mice and 5% (1/20, $p$>0.05) of the females did not survive the 39 weeks of 2-AAF exposure. The human non-genotoxic carcinogen CsA caused a decrease in survival of the male *Xpc*p53* mice only (35%, 7/20, $p$<0.05); all wild type males survived the full exposure duration. Short-term exposure to the human non-genotoxic carcinogen DES induced a small reduction (10%, 2/20, $p$>0.05) in survival of wild type female mice, while the survival of the *Xpc*p53* females was dramatically decreased (89%, 17/19, $p$<0.05). The low potent genotoxic carcinogen Phe and rodent non-genotoxic carcinogens DEHP and WY had no or only a slight (non-significant, $p$>0.05) effect on survival of both wild type and *Xpc*p53* mice.

### *Increased cancer response in Xpc*p53 mice after GTXC and NGTXC exposure*

Comprehensive pathological analyses were performed on all known target tissues and all gross lesions of all wild type and *Xpc*p53* mice to assess possible tumor development due to exposures. Results of these analyses are shown in Table 2A (males) and 2B (females). *Xpc*p53* mice developed neoplastic disease, identifying several of the genotoxic and non-genotoxic carcinogens tested here, after 39 weeks of exposure. Incidences of neoplastic lesions upon 2-AAF, CsA and DES exposure were highly increased ($p$<0.001) when compared to their untreated controls. The potent genotoxicant 2-AAF resulted in a 100% and 75% tumor incidence in *Xpc*p53* male and female mice respectively ($p$<0.001). Not only genotoxic carcinogen exposure caused elevated tumor incidences, also the non-genotoxic carcinogens CsA and DES induced tumors in very high percentages of *Xpc*p53* mice (60% and 72% respectively), this in contrast to wild type mice (10% and 15%, respectively). Wild type mice did not reach significant levels ($p$>0.001) of tumor incidence upon 2-AAF, CsA or DES exposure. The tumor incidence for WY was too low to reach significant levels in both genotypes. However, a significant ($p$<0.001) increase in preneoplastic lesions (e.g. hyperplasic or eosinophillic foci of cellular alteration) was found in livers of *Xpc*p53* mice, while this was not the case in wild type mice. Finally, the duration and doses used for phenacetin and DEHP appeared to be insufficient to significantly induce a tumor response in both wild type and *Xpc*p53,* which is also in line with our previous short-term carcinogenicity studies using wild type and *Xpa*p53* mice.

**A.**

| | Wild type | | | | Xpc*p53 | | | |
|---|---|---|---|---|---|---|---|---|
| **Exposure** | **Untreated** | **2-AAF** | **Phe** | **CsA** | **Untreated** | **2-AAF** | **Phe** | **CsA** |
| **Total no. mice** | 40 | 20 | 20 | 20 | 40 | 18 | 20 | 20 |
| **TBA (%)** | 1 (3%) | 7 (35%) | 0 (0%) | 2 (10%) | 5 (13%) | 18 (100%*) | 0 (0%) | 12 (60%*) |
| | | | | | | | | |
| **Liver** | **1 (3%)** | **4 (20%)** | **0** | **0** | **0** | **3 (15%)** | **0** | **0** |
| hep cell adenoma | 1 | 3 | - | - | - | - | - | - |
| hep cell carcinoma | - | 1 | - | - | - | - | - | - |
| cholangioma | - | - | - | - | - | 3 | - | - |
| preneoplastic FCA | - | 1 | - | - | - | - | - | - |
| **Urinary Bladder** | **0** | **1 (5%)** | **0** | **0** | **0** | **18 (100%)** | **0** | **0** |
| papilloma | - | - | - | - | - | 1 | - | - |
| carcinoma | - | 1 | - | - | - | 16 | - | - |
| fibrosarcoma | - | - | - | - | - | 1 | - | - |
| preneoplastic FCA | 2 | 9* | - | - | 2 | - | - | - |
| **Hematopoietic** | **0** | **2 (10%)** | **0** | **1 (5%)** | **5 (13%)** | **4 (20%)** | **0** | **12 (60%)** |
| lymphoma | - | 2 | - | 1 | 4 | 3 | - | 9 |
| histiocytic sarcoma | - | - | - | - | 1 | 1 | - | 3 |
| leukemia | - | - | - | - | 1 | - | - | 1 |
| preneoplastic FCA | - | - | - | 18* | 2 | - | - | 17* |
| **Other** | **0** | **0** | **0** | **1 (5%)** | **0** | **0** | **0** | **0** |
| hemangioma | - | - | - | 1 | - | - | - | - |

**B.**

| | Wild type | | | | | Xpc*p53 | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Exposure** | **Untreated** | **2-AAF** | **WY** | **DES** | **DEHP** | **Untreated** | **2-AAF** | **WY** | **DES** | **DEHP** |
| **Total no. mice** | 40 | 20 | 20 | 20 | 20 | 40 | 20 | 20 | 18 | 19 |
| **TBA (%)** | 0 (0%) | 1 (5%) | 4 (20%) | 3 (15%) | 3 (15%) | 5 (13%) | 15 (75%*) | 5 (25%) | 13 (72%*) | 0 (0%) |
| | | | | | | | | | | |
| **Liver** | **0** | **0** | **4 (20%)** | **0** | **0** | **3 (8%)** | **13 (65%)** | **5 (25%)** | **1 (6%)** | **0** |
| hep cell adenoma | - | - | 4 | - | - | 1 | 10 | 5 | - | - |
| hep cell carcinoma | - | - | - | - | - | - | 3 | - | 1 | - |
| cholangioma | - | - | - | - | - | 2 | - | - | 1 | - |
| preneoplastic FCA | - | 2 | 4 | - | - | - | 2 | 10* | - | - |
| **Urinary Bladder** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** |
| papilloma | - | - | - | - | - | - | - | - | - | - |
| carcinoma | - | - | - | - | - | - | - | - | - | - |
| fibrosarcoma | - | - | - | - | - | - | - | - | - | - |
| preneoplastic FCA | 3 | 3 | - | - | - | 2 | 2 | - | - | - |
| **Hematopoietic** | **0** | **1 (5%)** | **0** | **2 (10%)** | **3 (15%)** | **2 (5%)** | **4 (20%)** | **0** | **4 (22%)** | **0** |
| lymphoma | - | 1 | - | 2 | 3 | 2 | 3 | - | 4 | - |
| histiocytic sarcoma | - | - | - | - | - | - | - | - | - | - |
| leukemia | - | - | - | - | - | - | 1 | - | - | - |
| preneoplastic FCA | - | - | - | - | - | - | - | - | - | - |
| **Bone** | **0** | **0** | **0** | **1 (5%)** | **0** | **0** | **0** | **0** | **9 (50%)** | **0** |
| osteosarcoma | - | - | - | - | - | - | - | - | 6 | - |
| histiocytic sarcoma | - | - | - | - | - | - | - | - | 2 | - |
| bone marrow lymphoma | - | - | - | 1 | - | - | - | - | 1 | - |
| **Pituitary** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **0** | **2 (11%)** | **0** |
| adenoma | - | - | - | - | - | - | - | - | 2 | - |
| **Other** | **0** | **0** | **0** | **0** | **0** | **1 (3%)** | **2 (10%)** | **0** | **0** | **0** |
| osteosarcoma | - | - | - | - | - | 1 | 2 | - | - | - |

**Table 2.** Neoplastic and preneoplastic lesions after carcinogen exposure in *Xpc*p53* and wild type male (**A**) and female (**B**) mice. Summarized neoplastic and preneoplastic findings of short-term carcinogenicity studies in wild type and *Xpc*p53* mice. The total number of animals per exposure group, plus the number and percentage of tumor bearing animals (TBA) are shown in the upper part (grey) of the table. Some tumor bearing animals had more than one tumor. Underneath, specific tumor incidences per organ are depicted (grey rows). Tumor types and total number of preneoplastic FCA (foci of cellular alteration) are specified per organ at the bottom of each segment (white rows). * = $p \leq 0.001$ treated group versus corresponding controls (Fisher Exact test, one sided).

***Divergent non-neoplastic responses after GTXC exposure between Xpc*p53 and wild type mice***

Besides divergence in (pre-)neoplastic lesions between wild type and *Xpc*p53* mice, incidences of several non-neoplastic lesions differed between the two genotypes (Table 3). 2-AAF exposure, for example, induced a marked increase in hydronephrosis incidence in *Xpc*p53* male and female mice and not in wild type male or female mice. Similarly, a striking difference was observed after phenacetin exposure, causing increased hypertrophy and eosinophilia in liver, as well as pronounced tubular anisokaryosis in the kidney, a known target tissue of phenacetin. Here, *Xpc*p53* mice displayed a large increase for these types of lesions, while this response is absent in wild type mice. In general, the non-neoplastic lesions induced by exposure to genotoxic carcinogens were more severe in *Xpc*p53* mice than in wild type mice, while most non-neoplastic lesions after NGTX carcinogen exposure were of a comparable degree in both genotypes.

Increased incidence of hydronephrosis, for instance, was also observed after DEHP and DES exposure, but at similar incidences in both genotypes. Although in a progressed stage this is a harmful lesion, in the present study no clinical effects were observed. Several non-neoplastic lesions confirmed the compound exposures, like for example the increase in periarteriolar lymphocyte sheath (PALS) depletion in spleen and Kupffer cell activation in liver upon CsA exposure. CsA is known to interfere with normal immune surveillance [19]. Hyperostosis and fibrous osseous lesions are classical findings related to DES exposure [17] and are considered predictive of the bone tumor induction exhibited in this and other studies. The increase of lipofuscin accumulation upon DEHP exposure is also a classical response [23], which can be explained by the enhanced levels of oxidative stress caused by DEHP exposure and subsequent biotransformation.

| Carcinogen | Tissue | Sexe | Observation | WT | XpcP53 |
|---|---|---|---|---|---|
| **2-AAF** | Liver | M | Hypertrophy and eosinophilia | = | +++++ |
| | | M | Kupffer cell activation | = | ++++ |
| | Kidney | F | Hydronephrosis | = | + |
| | | M | Hydronephrosis | = | +++ |
| | | F | Tubular anisokaryosis | - | ++ |
| **Phe** | Liver | M | Lipofuscin | +++ | +++ |
| | | M | Hypertrophy and eosinophilia | + | +++++ |
| | Kidney | M | Tubular anisokaryosis | = | ++++ |
| **CsA** | Liver | M | Kupffer cell activation | ++ | +++ |
| | | M | Oval cell proliferation | + | + |
| | Spleen | M | Pals depletion/expansion | +++++ | ++++ |
| | | M | Lympholysis | +++ | = |
| | Mesenterial lymph nodes | M | Lympholysis | - | --- |
| | Thymus | M | Cortical apoptosis | + | = |
| **DES** | Liver | F | Anisokaryosis | - | - |
| | | F | Oval cell proliferation | + | +++ |
| | | F | Lymphohistiocytic aggregates | -- | --- |
| | Kidney | F | Hydronephrosis | ++ | +++ |
| | Femur | F | Fibrous osseous lesions | +++ | +++++ |
| | | F | Hyperostosis | ++++ | +++++ |
| | Uterus | F | Stromal collagen deposistion | +++++ | = |
| | | F | Stromal hyperplasia | +++++ | + |
| | | F | Atrophic uterus | +++ | +++++ |
| | Pituitary | F | Pars distalis degeneration | ++ | + |
| | | F | Angiectasis | +++ | ++ |
| **WY** | Liver | F | Anisokaryosis | - | - |
| | | F | Pigmented macrophages | +++++ | +++++ |
| | | F | Oval cell proliferation | ++++ | +++++ |
| **DEHP** | Liver | F | Lipofuscin | +++ | +++ |
| | Kidney | F | Hydronephrosis | +++++ | +++++ |

**Table 3.** Overview of most notable changes in non-neoplastic lesion incidence or severity upon carcinogen exposure in wild type and *Xpc*p53* mice, compared to the untreated mice within its own genotype. An increase or decrease in lesion incidence or severity is scored ranging from minor (+ or -) to severe (+++++ or - - - - -) respectively. No change observed (=).

### *Divergent genotoxic response between primary hepatocytes of Xpa*p53 and Xpc*p53 mice*

Previous studies showed that genotoxic substances are more toxic to *Xpa*-deficient mice than *Xpc* mice [9]. Since the doses used in the present study were based on previous dose range finding studies with *Xpa* or *Xpa*p53* mice, some of the substances tested in our current study with *Xpc*p53* mice could possibly have been dosed higher. For the assessment of carcinogenic risk it would be beneficial if

the higher (but tolerated) dosages used for wild type mice can also be used in the alternative testing model, without inducing a cytotoxic response. As a first step to compare toxicity between the genotypes, we tested cytotoxicity dynamics upon exposure to several carcinogens in primary mouse hepatocytes of all three genotypes. For this purpose we used the colorimetric MTT assay (Figure 3) and tested cell survival upon exposure to several carcinogens. We tested two non-genotoxic carcinogens that were also used in our *in vivo* studies; CsA and WY. Mitomycin C and Aflatoxin B1 were selected as GTX carcinogens, since the solubility of 2-AAF is very poor and phenacetin is a very weak genotoxic compound. Results (see Fig 3, E-H) indicated that *Xpc*p53* primary hepatocytes follow the cytotoxicity dynamics of wild type cells. Hepatocytes isolated from these two genotypes are equally tolerant to high doses of the genotoxic carcinogens tested, whereas on average, *Xpa*p53* hepatocytes are approximately threefold more sensitive (compare Fig 3A, B with Fig 3E, F). All three genotypes tested showed a comparable response to the non-genotoxic carcinogens WY and CsA is comparable between all three genotypes tested (see Fig 3C, D, G, H).

## Discussion

Society would benefit from a more accurate, speedier, less expensive alternative model for carcinogenicity testing that also addresses the three R's of Russell and Burch. Ideally, such a test system would combine broad applicability with high prediction accuracy for the human situation. To achieve this full cell, organ and systemic functioning (*e.g.* functional biotransformation, immune response and signal transduction) is preferred, if not required. Secondly, the model should be suited to test all substances in a relevant dosage, preferably mimicking human exposure routes (*e.g.* solid, solved or volatile substances that are administrated orally, by inhalation or contact).

We previously demonstrated that the *Xpa*p53* mouse model can predict carcinogens with both genotoxic and non-genotoxic modes of action after only a 39 weeks of exposure. *Xpa*p53* mice, however, suffer from enhanced toxicity when exposed to, in particular, genotoxic carcinogens, making them less attractive to use in quantitative cancer risk assessment. Therefore, we tested whether the *Xpc*p53* mouse model is a more suitable model.

Whilst covering the abovementioned preferable traits of an alternative carcinogenicity test system, our studies showed promising results. The *Xpc*p53* mouse model not only responded to genotoxic, but also to non-genotoxic carcinogens. In contrast to wild type mice, a very strong and significant tumor response was observed for the genotoxicant 2-AAF and the non-genotoxic human carcinogens CsA and DES in *Xpc*p53* mice. Unexposed *Xpc*p53* mice had a low spontaneous tumor incidence (13%), which is comparable to the low incidence of 11% previously found in the *Xpa*p53* mouse model in a 39-week exposure setting [6]. Exposure to 2-AAF resulted in a strong increase in tumor bearing animals compared to the *Xpa*p53* model (100% versus 53% respectively in male mice, 75% versus 60% in female mice) [6]. A large portion of the *Xpc*p53* mice (100% of the male mice, 45% of the female mice) did not survive the 39 weeks of 2-AAF exposure. These animals died from life-threatening tumors, which occur apparently at lower frequencies in 2-AAF-treated *Xpa*p53* mice [24].

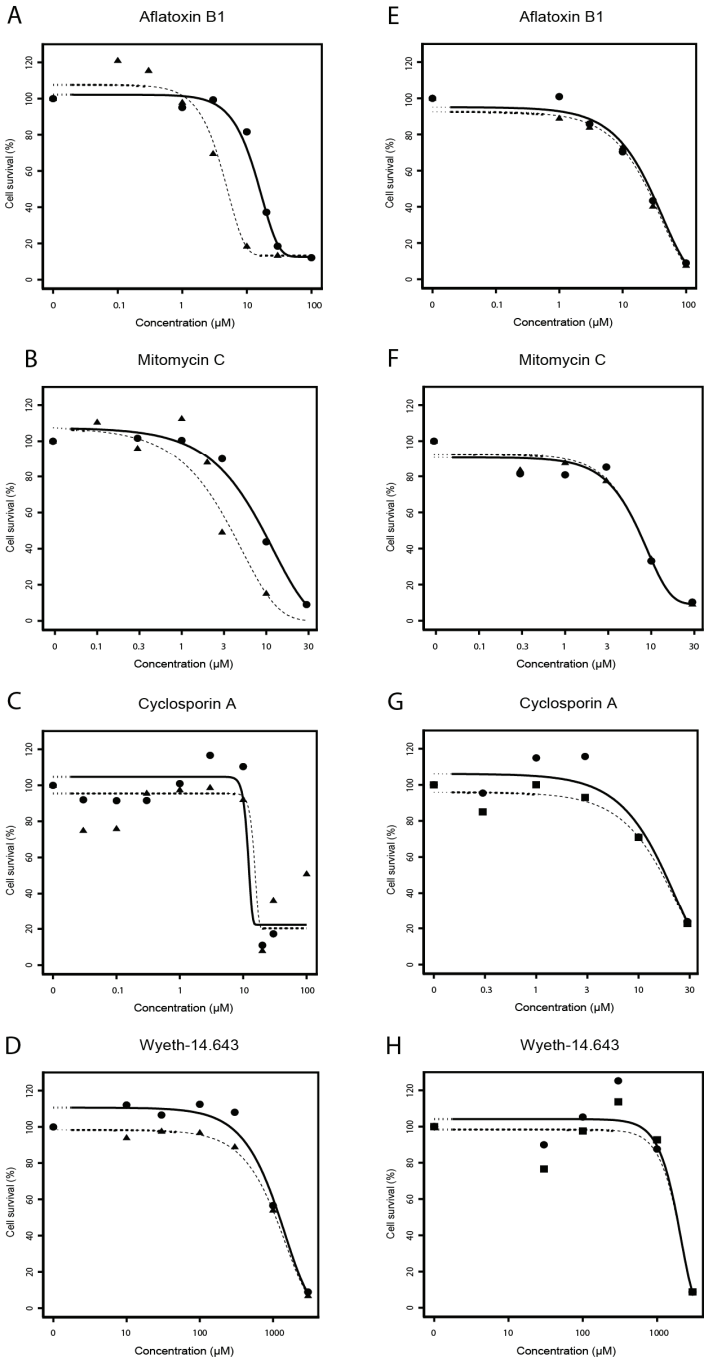



**Figure 3.** Cytotoxicity (MTT) dynamics of primary hepatocytes upon exposure to a dose-range of genotoxic (AFB1 and MMC) and non-genotoxic (CsA and WY) carcinogens. Panel **A-D** show the response of exposed wild type (●) versus *Xpα*p53* (▲) cells. Panel **E-H** show the response of exposed wild type (●) versus *Xpc*p53* (■) cells.

Exposure to DES in *Xpc*p53* mice also resulted in an increase in tumor induction compared to *Xpa*p53* and wild type mice (72% versus 47% versus 15% respectively) [6]. Other alternative *in vivo* models, like the Tg.AC and the neonatal models for carcinogenicity testing, did not identify this substance as a carcinogen [25;26]. The *p53*$^{+/-}$ mouse model did also identify CsA and DES as a carcinogen, which indicates the heterozygous state of p53 could be a key factor in the carcinogenic response. CsA and DES are regarded as non-genotoxic carcinogens and are believed to facilitate tumor promotion, instead of tumor initiation. It is our hypothesis that the higher spontaneous mutational load in both XP models, detected in our previous studies [27], will trigger tumor initiation. Accumulation of initiated cells could be the underlying principle in *Xpa*p53* and *Xpc*p53* mice of the observed tumor proneness in response to exposure to non-genotoxic carcinogens like CsA and DES.

WY exposure showed a relatively weak carcinogenic response in both wild type and *Xpc*p53* mice, although a significant increase in preneoplastic lesions was found for the latter genotype. The 39 weeks of WY exposure was not long enough to undoubtedly demonstrate the carcinogenic potential of this compound, but also demonstrates WY is a relatively weak non-genotoxic rodent carcinogen, possibly harmless to humans. DEHP shares part of its mode of action with WY, namely peroxisome proliferation [23;28]. Even though the applied dose of DEHP in this study was much higher than that of WY, it did not significantly induce cancer in wild type or *Xpc*p53* mice, which is consistent with previous studies [6].

Exposure to the low potent genotoxic carcinogen phenacetin, did not result in identification of the compound as a carcinogen in wild type, *Xpa*p53* or *Xpc*p53* model in our studies. Previously, 75-80 week exposures to phenacetin in female and male wild type (C57BL/6) mice did not result into any tumor induction [29;30]. Additionally, previous 39-week exposures to phenacetin using *Xpa* and *Xpa*p53* mice did not result in an increased tumor response [6;18]. Other alternative *in vivo* models (*p53*$^{+/-}$, Tg.AC and neonatal carcinogenicity model) plus several *in vitro* tests (e.g. SHE cell transformation assay, DNA-repair test) were also not able to identify this carcinogen [25;31;32]. Interestingly, although phenacetin is a (very weak) substrate to NER [33], it does not give rise to an enhanced tumor response even in a NER-deficient genetic setting. It is possible that phenacetin is only harmful at extremely high doses. In our present study the phenacetin dose used was equal to the highest dose of phenacetin used in the dose range finding study performed with *Xpa* and *Xpa*p53* mice [18]. Possibly, wild type and *Xpc*p53* mice can be dosed higher without interference of a (geno)toxic response, as observed *Xpa* or *Xpa*p53* mice.

In the past, Wijnhoven et al. have demonstrated an increased sensitivity of *Xpa,* as compared to *Xpc* mice, towards toxic effects induced by DNA-damaging agents [9]. Additional *in vivo* data from our lab (unpublished results) supported that *Xpa* mice are more sensitive towards genotoxicant-induced cytotoxicity than *Xpc* mice, due to a defective TC-NER (in addition to GG-NER defect). The *Xpa* mice exhibited a strong apoptotic response, resulting in cell death [8;34-36]. Our current study shows that primary mouse hepatocytes derived from *Xpc*p53* mice can be exposed to higher doses of genotoxicants than *Xpa*p53* cells, before cytotoxicity occurs. It appears the divergence in toxicity response between *Xpa* and *Xpc*-deficient systems is not altered when additional p53 heterozygosity is introduced. The same doses as wild type cells were tolerated by the *Xpc*p53*-derived hepatocytes. This was in contrast to the situation in *Xpa*p53*-derived cells, which appeared to be approximately threefold more sensitive to genotoxic carcinogens than wild type cells. As *Xpc*p53* cells responded similarly to genotoxicant-induced cellular toxicity as wild type cells, we hypothesize that *Xpc*p53* and

wild type mice will exhibit similar dose-responses in (short-term) carcinogenicity testing although this assumption needs further validation.

As noted, the *Xpc*p53* mice have an additional advantage over *Xpa*p53* mice since they are supposed to be not only sensitive to NER-inducing compounds, but also to other DNA-damaging agents. We showed previously [27] that *Xpa* and *Xpc* mice have divergent tumor phenotypes in this respect. Therefore, we conclude that the *Xpc*p53* mouse model is a promising potential alternative tool for carcinogenicity testing. The deficiency of XPC (like XPA) also facilitates the most common enabling characteristic of carcinogenesis, namely genomic instability and is, therefore, suitable to predict both true genotoxic as well as non-genotoxic carcinogens, which several other alternative *in vivo* carcinogenicity models are incapable of. Using this beneficial feature, more carcinogenic compounds should be tested *in vivo* to ensure that the *Xpc*p53* is a reliable and accurate alternative candidate in short-term carcinogenicity testing.

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## Conflict of Interest Statement

None declared.

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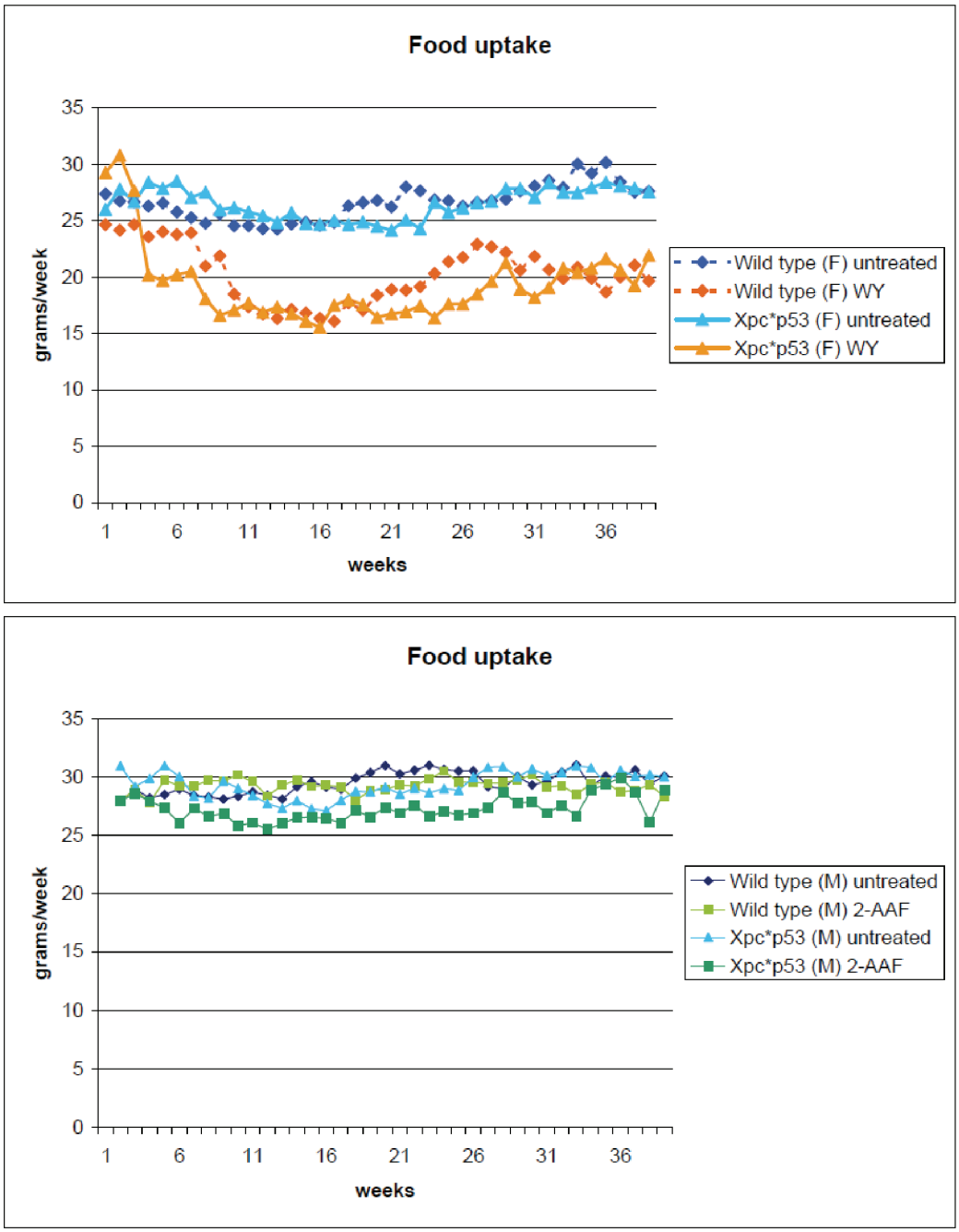


**Supplemental information 1.** Food uptake (weekly average uptake per mouse) of wild type and *Xpc*p53* mice exposed to control feed and WY (females, **A**) or 2-AAF (males, **B**).