Supplementary Material

Irradiation conditions for the X-ray pilot study

For determining the correct dose for the proton study, a pilot study was performed using BALB/c mice that were irradiated with X-rays ranging from 0 Gy (sham irradiation) up to 60 Gy (6 mice per group). X-ray broad beam irradiations were conducted at the RS225 (X- Strahl Ltd) of the *clinics X*. 70 kV X-rays were administered to the right ear of the mice through a 7.2 x 7.2 mm^2 tungsten collimator, using doses of 0, 2, 5, 10, 20, 40 and 60 Gy (calibrated with radiochromic films and a reference ionization chamber; dose uncertainty ~ 6 %). During irradiation, anaesthetized mice were lying on their back on a specially designed holder, with the right ear fixed on an elevated plateau of the holder. The mouse was protected from radiation by 3 mm tungsten, leaving only the ear uncovered. The tungsten collimator was mounted on the ear plateau such that the square hole for irradiation was above the center of the ear, shielding the rest of the ear from radiation. Spatial dose distributions were analyzed using radiochromic films ~ 2 days after irradiation (Gafchromic EBT3, cf. (1)). The different direction of the beam towards the ear compared to the proton irradiations, where the beam first hits the inside of the ear, is negligible due to the small thickness of the ear, which allows the dose to be approximated as constant throughout the whole ear thickness of 250 µm (dose decrease through ear < 3 % for 20 MeV protons and ~ 5 % for 70 kV X-rays).

X-ray pilot study for dose determination

The pilot study using broad beam X-ray irradiation of the mouse ear was required for determination of a dose effect curve of the used model as a reference for the proton minibeam study. Inflammatory responses, such as ear swelling and visible skin reactions were monitored within a follow-up period 90 days after irradiation.

No ear swelling or any other morphologically visible skin reaction was detected for doses of 5 Gy and below during the 90 days observation time (cf. Fig. S1). An irradiation with 20 Gy resulted in a minor increase in the ear thickness of < 100 µm about 4 weeks after irradiation. At the same time after irradiation with 40 and 60 Gy the thickness of the ear with an initial size of 260 µm increased up to 4-fold. Significant erythema and desquamation only developed in ears irradiated with doses ≥ 40 Gy (cf. Fig. S2). The peak skin reaction was similar after 40 and 60 Gy, but was reached 1-2 days earlier after a dose of 60 Gy. Hair loss in the irradiation field was first visible 4-5 weeks after irradiation with doses > 40 Gy. Therefore, an average dose of 60 Gy leading to significant and measurable inflammation after X-ray irradiation was chosen for proton minibeam and homogeneous irradiation.

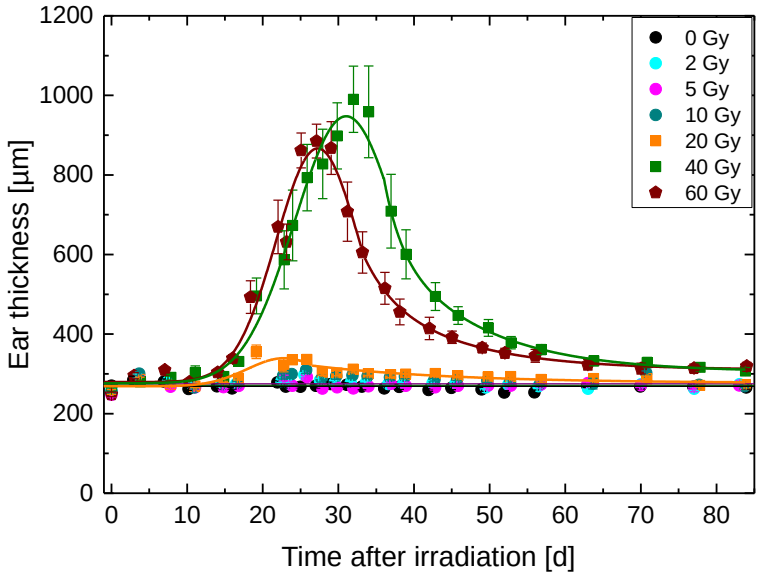

Fig. S1 Ear thickness after X-ray irradiations (0-60 Gy).

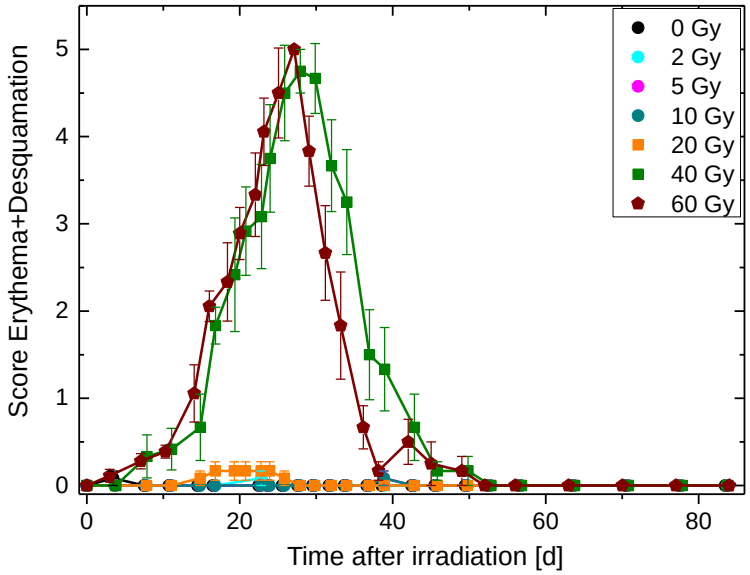

Fig. S2 Ear skin reactions after X-ray irradiations (0-60 Gy). The skin score is the sum of the erythema and the desquamation score, according to Table 1.

Irradiation conditions for the proton study

Two different beam preparation set-ups were used for homogeneous and minibeam irradiation and the same total number of counted protons (7.34×10^9) was applied within an area of 51.84 mm^2 resulting in a mean dose of 60 Gy. For the homogeneous irradiation mode this was achieved with the microprobe's slit system, allowing the initially wide beam to be cut down to the desired dimensions without further focusing. Minibeams were produced by scanning a focused microbeam of roughly 1 µm size across a square area of 180 µm edge length (4.69×10^8 p/minibeam). The resulting dose within the minibeams, which cover about 1 % of the total area, is approximately 6,000 Gy, while the dose in between the irradiated beams approaches zero (< 0.6 Gy for 93 % of the area, cf. *ref. 2*).

Ear thickness measurements after proton irradiations

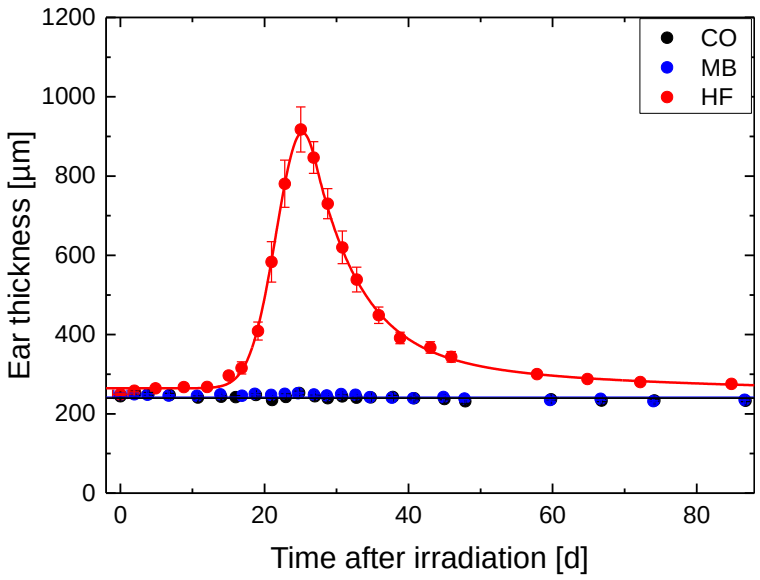

Fig. S3 Ear thickness after proton minibeam (MB) and homogeneous (HF) irradiation, in comparison to sham-irradiated controls (CO).

Scoring of the skin reaction after proton irradiations

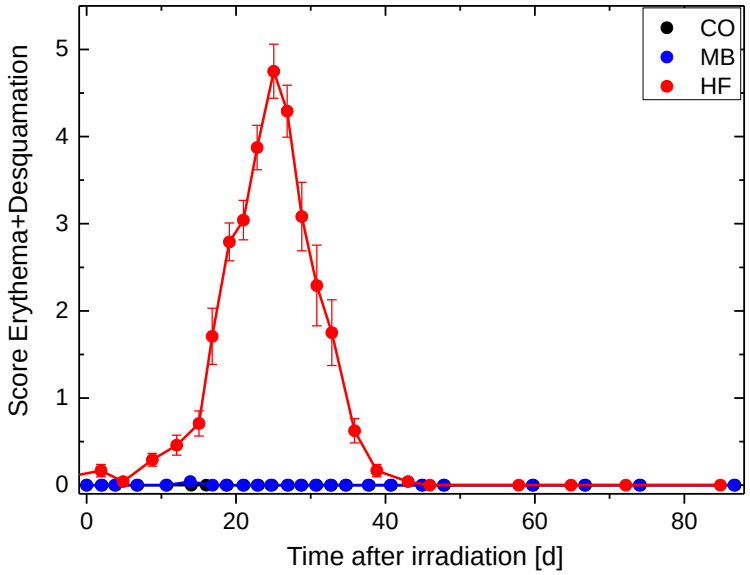

Fig. S4 Ear skin reactions after proton minibeam (MB) and homogeneous (HF) irradiation, in comparison with sham-irradiated controls (CO). The skin score is the sum of the erythema and the desquamation score, according to Table 1.

References

1. Reinhardt S, Hillbrand M, Wilkens JJ*, et al.* Comparison of Gafchromic EBT2 and EBT3 films for clinical photon and proton beams. *Medical physics* 2012;39 (8):5257–62.

2. *XXX*