Tracing the footprints of radiation: temporal and spatial patterns of γH2AX signaling in different human cells after exposure to X-rays and UV-C light

UNIVERSITÀ DI PAVIA
Dipartimento di Fisica "Alessandro Volta"

Isabella Guardamagna[1], Leonardo Lonati[1], Ombretta Iaria[1], Alice Mentana[1], Géraldine Gonon[1], Daniele Parodi[1], Giulia Peterlin[1], Rossella Semerano[1], Cecilia Riani[1], Andrea Previtali[2], Anna Tricarico[2], Paola Tabarelli de Fatis[3], Giovanni Battista Ivaldi[4], Paola Perucca[2], Ornella Cazzalini[2], Giorgio Baiocco[1]

[1]Laboratory of Radiation Biophysics and Radiobiology, A. Volta Department of Physics, University of Pavia, 27100 Pavia, Italy.
[2]Unit of Immunology and General Pathology, Department of Molecular Medicine, University of Pavia, 27100 Pavia, Italy.
[3]Unit of Medical Physics, ICS Maugeri, IRCCS, 27100 Pavia, Italy.
[4]Unit of Radiation Oncology, ICS Maugeri, IRCCS, 27100 Pavia, Italy

Contact: isabella.guardamagna@unipv.it

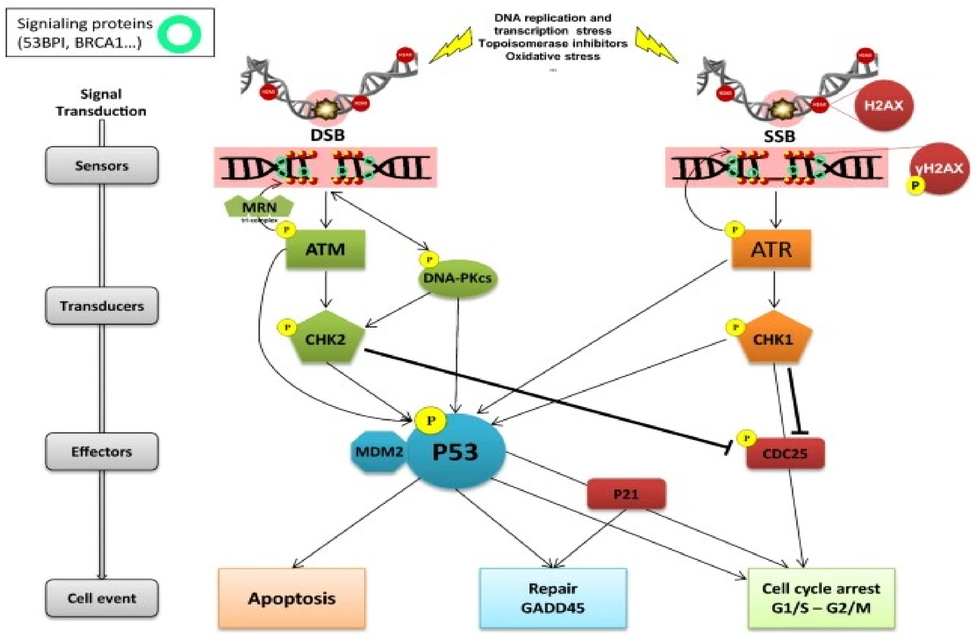

Figure 1: Taken from "Kopp, Bet al., Validation of the γH2AX biomarker for genotoxicity assessment: a review. (2019)"

Background

γH2AX is widely used as a biomarker of DNA damage. More precisely, phosphorylation of histone H2AX at serine 139 is a post-translational modification triggered by **DNA strand breaks or by distortions of the DNA helix**. For this reason, γH2AX should be regarded not as a direct marker of damage itself, but rather as a **sensor of the DNA damage response (DDR)**. As a consequence, γH2AX activation is strongly dependent on the **efficiency of damage processing and repair** of the biological system under investigation, as well as on the **cell-cycle phase**. Indeed, H2AX phosphorylation is mediated by kinases such as ATM and ATR, which play a well-defined role in cell-cycle regulation and checkpoint control (Fig. 1). γH2AX is often interpreted as a specific signal of DNA double-strand breaks. However, the molecular mechanisms leading to its activation are **not uniquely determined by the type of DNA lesion**, but rather by the downstream signaling pathways engaged in response to genotoxic stress.

What is the biological interpretation of γH2AX activation, and which techniques and data analysis approaches allow us to extract the maximum amount of information from its activation?
The most common radiobiological approach relies on counting γH2AX foci using imaging techniques. However, this is not the only possible strategy, as foci counting does not fully describe the underlying biological response and, most importantly, it is not applicable to all genotoxic agents.

Experimental approach

We selected three human cell lines with different genetic backgrounds and different clonogenic responses to X-ray irradiation (Fig. 2):
- **HaCaT**: immortalized human keratinocytes
- **Caco-2**: colorectal adenocarcinoma
- **HeLa**: cervical adenocarcinoma

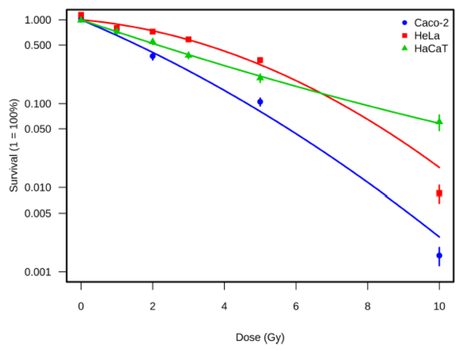

Figure 2: clonogenic survival of the three different cell lines exposed to X-ray (experimental data and best-fit with the LQ model)

Two different genotoxic agents were employed, both inducing damage through **photon-mediated energy deposition**: X-rays and UV-C radiation.
Experimental setups were optimized to ensure controlled and reproducible exposure conditions, allowing a defined amount of energy to be deposited in the biological sample, quantified in **Gy for X-rays** and **J/m² for UV-C**.

Conclusions

Our results highlight that γH2AX activation represents a common biological response to photon-mediated genotoxic stress, but its spatial and temporal characteristics are strongly shaped by radiation quality and by the way radiation interacts with biological matter. UV-C exposure is known to induce relatively simple DNA distortions, such as cyclobutane pyrimidine dimers (CPDs) and (6–4) photoproducts, and one might expect a rapid resolution of γH2AX signaling. However, our data demonstrate that γH2AX dephosphorylation after UV-C exposure is strongly cell-type dependent, indicating that repair efficiency is not determined solely by lesion complexity, but critically depends on the intrinsic biological properties of the cell. Overall, these findings emphasize that γH2AX should be interpreted as a functional sensor of the DNA damage response rather than as a direct marker of specific DNA lesions, and that both radiation quality and cellular context must be considered for a correct quantitative interpretation of γH2AX-based measurements.

Thesis on this topic

- **Bianca del Giudice**, Bachelor's degree in Physics (L-30): Danno al DNA indotto da raggi X e radiazione UV: meccanismi fisici e misure radiobiologiche.
- **Daniele Parodi**, Master's degree in Physical Sciences (LM-17): Quantificazione del γH2AX come marcatore di danno al DNA indotto da raggi X e UV-C tramite software di analisi immagini Fiji/ImageJ.
- **Rossella Semerano**, Master's degree in Experimental and applied Biology (LM-6): Metodi di analisi e quantificazione del γ-H2AX come marcatore di danno al DNA indotto da raggi X e UV-C.
- **Elena Piccinni**, Bachelor's degree in Biology (L-13): Quantificazione del danno al DNA da radiazioni UVC mediante marcatori γ-H2AX e CPD in linee cellulari umane.
- **Giulia Peterlin**, Master's degree in Physical Sciences (LM-17): DNA damage and repair after UV-C exposure of different *in vitro* systems: fluorescence microscopy and image analysis applied to gain molecular insight on the correlation between γ-H2AX and CPDs signals.

Results

Using two genotoxic agents that induce DNA damage through **photon-mediated energy deposition** (X-rays and UV-C) we observed: activation of γH2AX signaling. This result highlights that both ionizing and non-ionizing radiation are able to trigger the DNA damage response (Fig. 3). However, the different nature of the radiation becomes evident at the biological level when first analysing the **spatial distribution** of the γH2AX signal. Fluorescence microscopy images reveal a classical **foci-like nuclear localization** after X-ray exposure, consistent with sparse energy deposition and discrete damage sites. In contrast, UV-C exposure results in a predominantly **pan-nuclear γH2AX distribution**, and, if foci-like structures can still be visually identified, their density is so high that discrete foci counting is no longer feasible.

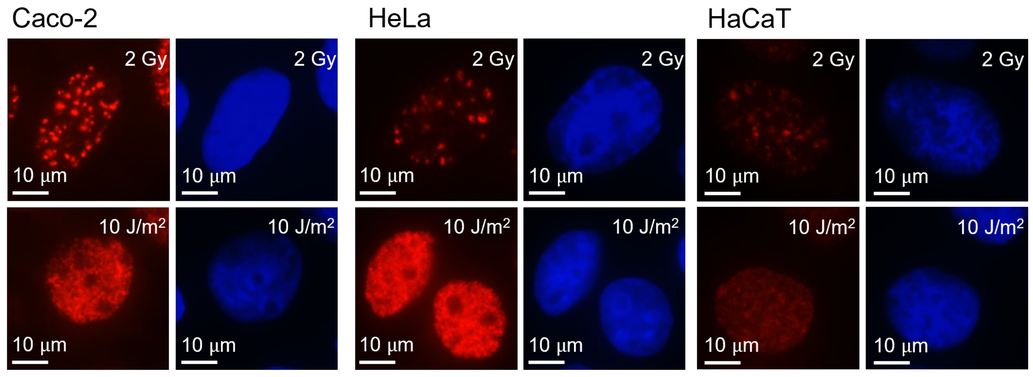

Figure 3: representative fluorescence microscopy images of γH2AX signal (in red) in cell nuclei (DNA in blue), after exposure to 2Gy X-rays or 10 J/m2 UV-C.

A quantitative analytical approach to compare the biological effect across radiation qualities was required. γH2AX activation was evaluated by dose-response analysis using 0.5, 1, 2, and 5 Gy for X-rays and 10, 20, and 40 J/m² for UV-C irradiation. A sham-treated sample was consistently included as a non-irradiated control. We therefore quantified γH2AX activation in terms of integrated nuclear fluorescence expressed **as mean fluorescence intensity**. To exclude possible imaging-related limitations, such as signal saturation observed at higher exposure levels (Fig. 4), samples in the same conditions were analysed by flow cytometry (Fig. 5), expressing γH2AX as **median fluorescence intensity**. The consistency between microscopy and cytometric data demonstrates that the observed saturation behavior is **not technique-dependent**, rather reflects an intrinsic **biological response**. Analyzing the kinetics of γH2AX activation (20, 30, and 40 min; 1, 2, 4, 6, and 24 h), we observed that γH2AX activation is **strongly dependent on radiation quality**. Indeed, radiation quality determines how energy is deposited to biological matter, ultimately shaping the type of DNA lesions produced and the dynamics of the DNA damage response.
Different radiation qualities induce **different types of DNA damage or structural distortions**, leading to radiation-specific γH2AX activation and resolution kinetics. In particular, the **signal decay phase** is highly characteristic and reflects the efficiency of damage processing and repair (Fig. 6).

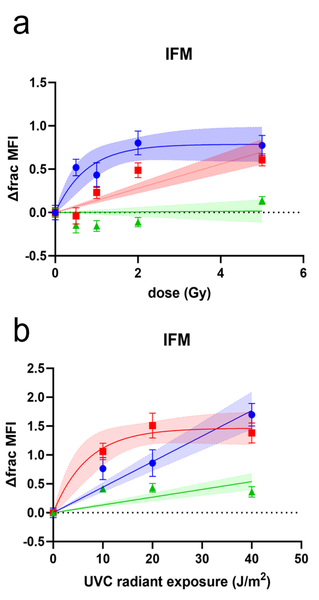

Figure 4: γH2AX dose-response quantification from microscopy images expressed as mean fluorescence intensity (MFI), fractional increase with respect to the control. A) samples exposed to X-rays. B) samples exposed to UV-C.

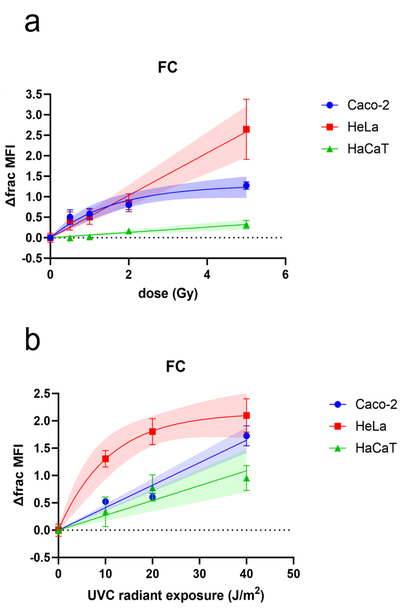

Figure 5: γH2AX dose-response quantification by flow cytometry expressed as median fluorescence intensity (MFI), fractional increase with respect to the control. A) samples exposed to X-rays. B) samples exposed to UV-C.

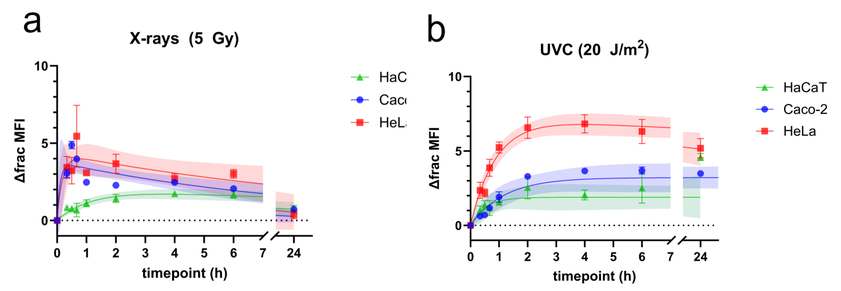

Figure 6: γH2AX kinetics quantification by flow cytometry expressed as median fluorescence intensity (MFI), fractional increase with respect to the control. A) samples exposed to 5 Gy X-ray. B) samples exposed to 20 J/m2 UV-C.

Moreover, γH2AX signaling in UV-C exposed samples remains activated for extended periods of time (up to 24h after irradiation). This could suggest that the measured signal arises both from viable cells actively attempting to repair DNA damage and from cells that fail to downregulate γH2AX activation and subsequently undergo programmed cell death (Fig. 7). However, quantitative biparametric analysis combining γH2AX detection with a cell-death marker demonstrates that the contribution of dead cells to the overall γH2AX signal is not significant (Fig. 8).

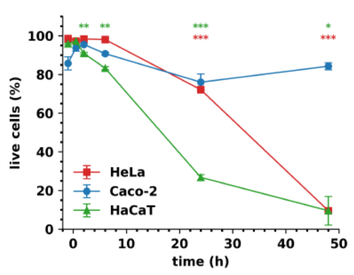

Figure 7: Percentage of viability as a function of time after 20 J/m2 of UV-C .

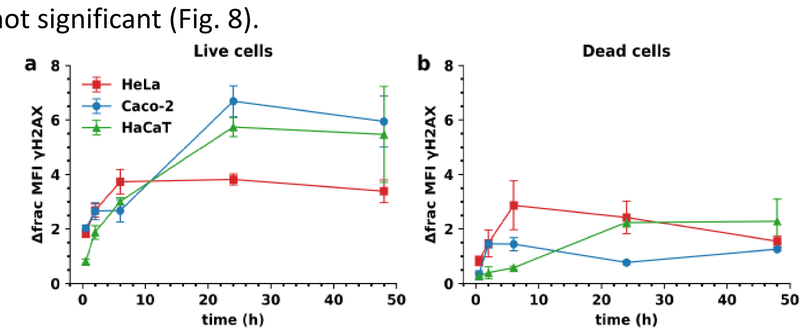

Figure 8: Biparametric analysis of γH2AX intensity and Live/Dead flow-cytometry assay after exposure to 20 J/m2 UV-C. A) viable cells. B) dead cells.