Formation of 5hmC following exposure to Ionizing Radiation *

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Eukaryotic DNA methylation (DNAm), which is the addition of a methyl group on cytosine bases (C), is an epigenetic mechanism involved in several biological processes, including replication, transcription, and carcinogenesis. Besides methylcytosine (5mC), hydroxymethylcytosine (5hmC) is another modified C moiety. 5hmC is formed by the enzymatic activity of TET (ten eleven translocation) enzymes oxidizing 5mC to 5hmC and further to formyl- and carboxyl-cytosine, the latter being finally replaced by C via base excision repair [1]. Conversion of 5mC to 5hmC could change the DNA binding affinity of several proteins and thus influence regulation of gene transcription [2]. Previous works reported that ionizing radiation (IR) and DNA double strand break (DSB) repair processes are able to induce changes of DNAm levels [3, 4, 5]. However, these studies were performed several population doublings after IR exposure and could not rule out replication-dependent DNAm changes. Whether the latter may take place also at short times after exposure to IR remains controversial. Increased DNA methylase levels at later times ($\geq$ 24 hours) after IR exposure have been recently reported [6]. These findings suggest that increased DNAm levels would not occur until DNA repair is accomplished. Based on such observation, we hypothesize that IR may rather reduce DNAm levels at earlier times by triggering enzymatic activities regulating such process, e.g. TET enzymes. Therefore, we examined DNAm changes within one replication cycle time frame after IR ($\leq$ 24 hours), focusing on DNA demethylation. Namely, we investigated 5hmC formation as an intermediate of DNA demethylation and whether this modification was involved in the DSB repair. We investigated human fibroblasts 30 minutes after irradiation with carbon ions and observed 5hmC formation along the ion trajectory. Both fluorescence microscopy images and intensity profiles indicate that 5hmC signal colocalizes with γH2AX (DSBs marker) (Fig. 1). Similar results were obtained upon irradiation with X-rays (Fig. 2A). Colocalization was statistically verified using intensity correlation analysis (ICA) [7]. Briefly, PDM (Product of the Differences from the Mean) values were calculated for each pixel in both channels. If the signals were positively correlated, this would result in a positive PDM value and, thus, in a positive correlation. Our results show that most PDM values are positive, displaying a moderate positive correlation between 5hmC and γH2AX at DSBs ($R_{Pearson}$ = 0.425, Fig. 2B).

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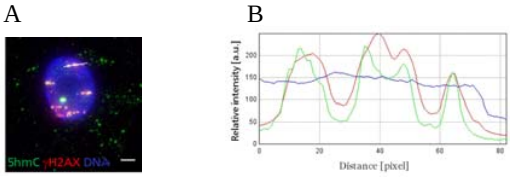

Figure 1: AG1522D cells were irradiated with carbon ions (LET: 170 keV/μm; fluence: 3x10^6 p/cm^2) under low angle, fixed after 30 minutes and stained against 5hmC (green) and γH2AX (red). DNA was stained with DAPI (blue). A) Micrograph showing 5hmC (green) formation at DSBs sites indicated by γH2AX (red). White line: ion trajectory; scale bar = 5 μm. B) Fluorescence intensity profiles showing colocalization of 5hmC (green) and γH2AX (red). DNA is illustrated by the blue line.

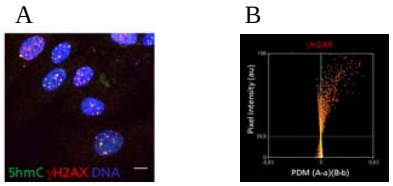

Figure 2: AG1522D human fibroblasts were irradiated with 0.5 Gy X-rays, fixed after 30 minutes and stained as in Fig. 1. A) 5hmC (green) accumulates at DSBs, indicated by γH2AX staining (red). Scale bar = 10 μm. B) ICA graph of γH2AX signal. The horizontal line indicates mean PFI values. With increasing PFI, PDM values increase.

Our results indicate that 5hmC is formed at DSB sites colocalizing with γH2AX, after both heavy ions and X-rays irradiation. Such observation suggests a possible involvement of 5hmC in the DSB repair. We are currently investigating the colocalization of 5hmC and γH2AX over time, during DNA repair, performing kinetics of foci formation in a time frame of $\leq$ 24 hours after irradiation.

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