Characterization of 53BP1 repair foci in the early response to a cluster of DNA double-strand breaks induced by CRISPR-Cas9 or visible light

Abstract

Maintaining genome stability is a fundamental feature of cells that ensures their survival and ability to pass on intact genetic material to their progeny cells. Despite the fact that thousands of different types of endogenous DNA damage occur per day, genes show remarkable sequence constancy and the mutation rate in normal cells is 10^{-10} per nucleotide per cell per generation. The cellular processes responsible for such low mutation rates have excellent accuracy, and most DNA damage is repaired without error. Endogenous damages to the genome resulting from normal cellular activity appears to be of little concern and, thanks to flawless repair mechanisms, are removed. However, any disruption of cellular activity resulting from endogenous factors, may result in the accumulation of harmful metabolites, free radicals or reactive oxygen species. This can promote the accumulation of DNA damage which, if not repaired, has serious consequences, leading to mutations, cell cycle arrest, carcinogenesis, activation of apoptosis, and eventually cell death. Cancer cells, in turn, are characterised by a number of abnormalities compared to normal cells, ranging from a dysregulated cell cycle, excessive metabolic activity and proliferation to a high number of mutations in the genome and abnormalities in the karyotype. Cancer cells may therefore exhibit differences in the DNA damage repair mechanisms relative to those in normal cells, so a thorough investigation of the activity of these processes is crucial for understanding the phenomenon of genome stability, the design of anti-cancer therapies and genetic diseases.

Of the many different types of DNA damage, double-stranded DNA breaks (DSBs) are the most dangerous for the maintenance of genome integrity, even though they represent a small percentage of all types of damage continuously induced in the cell. The mechanisms of repair of this damage are still being intensively studied and, so far, there are no clear conclusions regarding the activation of repair pathways and the role of most proteins that control and regulate these processes. The protein 53BP1, which has been identified as a marker of non-homologous end joining (NHEJ) repair, was the subject of the studies described in this PhD thesis. The aim was to characterise the repair foci of the 53BP1 protein formed in the cellular response to DNA damage induced by the Cas9 endonuclease of the type II CRISPR system or

by a focused beam of laser light at a wavelength in the visible range. The CRISPR-Cas9 method allowed the induction of a cluster of double-stranded DNA breaks at known genome repetitive sequences in the subtelomeric region of chromosome 3 of U-2 OS cells. In contrast, the second method of DNA break induction using a focused beam of visible light allowed the local illumination of chromatin with a relatively low energy dose without the presence of photosensitisers and the induction of a small number of DNA breaks, mainly of both strands. Detection of 53BP1 foci was carried out using a confocal microscope, resulting in high-resolution images of single cells.

First, energy doses were optimised for three wavelengths of visible light: 458 nm, 488 nm and 561 nm required to effectively induce DNA double-strand breaks, the presence of which was identified indirectly by observing the accumulated 53BP1 protein focus at the irradiated site in HeLa cells. The study showed that a low energy dose of blue light (458 nm and 488 nm), > 25 μJ and 90 μJ, respectively, is sufficient to observe 53BP1 protein accumulation within a few minutes. In contrast, green light (561 nm) requires more than 200 times the energy dose of 20,000 μJ to have the same effect. It was also shown that the induction of DNA damage does not depend on the photon flux, but on the total energy dose delivered to the selected site in the cell nucleus.

Subsequently, the areas of maximum cross-sections of repair foci induced by visible light were calculated, ranging on average between 2 and 4.5 μm^2, with the largest foci observed after exposure of chromatin to a 488 nm laser light beam in HeLa cells. In contrast, repair foci formed in response to Cas9 endonuclease-induced DSBs in U-2 OS cells at maximum section occupied a much smaller area, averaging between 0.75 and 1.2 μm^2, although cases of larger 53BP1 foci of approximately 3.5 μm^2 were also recorded.

A surprising observation made when imaging control cells with inactive dCas9 endonuclease was to find a small percentage of cells in which co-localisation of the signal derived from the 53BP1 protein and other DSB repair markers (γH2A.X and BRCA1) with the dCas9 signal was observed. It is also interesting to note that usually the co-occurrence of these signals involved only one dCas9 focus.

The spatial correlation of the active endonuclease Cas9 and the 53BP1 protein (the co-occurrence of signals from the foci of both proteins), was determined as the number of events in which the intensity maxima of the foci were within 1 μm of each other. This was made possible by a computational algorithm developed in the Department of Cell Biophysics at the Jagiellonian University. Analysis of the distance between the fluorescence intensity maxima of the signals from Cas9 and 53BP1 showed that, on average, 3.6 out of 12 endonuclease foci

occur within 1 μm of the repair protein. An increased number of Cas9 foci were also observed than the number of potential target sites for the CRISPR system. These foci are characterised by a variety of signal intensities and are scattered throughout the volume of the cell nucleus. A similar analysis was also performed in cells into which two Cas9n nickases, which induce single-stranded DNA breaks on opposite strands, were introduced simultaneously. It turned out that the number of nickase foci corresponded to the average number of target sites (average number of chromosomes 3 in the genome), on average 2.5 foci out of 4 were correlating with the 53BP1 signal. Interestingly, a smaller number of 53BP1 foci that did not spatially correlate with the Cas9n nickase signal were also observed.

When simultaneously observing signals from CRISPR-Cas9 and protein pairs: 53BP1 and BRCA1, 53BP1 and PML, BRCA1 and PML. It appears that 53BP1 and BRCA1 and 53BP1 and PML signals often co-occur in the vicinity of the endonuclease focus, and that BRCA1 and PML do not show direct adherence of these signals despite the short distances between them and their non-coincidental location near Cas9. It is likely that 53BP1, as it forms the largest foci around DNA damage and is a major component of the repair foci acting as a molecular scaffold for other repair factors. It may surround the foci of both of these seemingly unrelated proteins. In addition, the intensity profiles of 53BP1 foci show that the highest signal density from 53BP1 is closer to the endonuclease foci, which may indicate that a certain minimum number of 53BP1 molecules are required to be accumulated at the site adjacent to the damage, and others may represent a fraction of the protein less associated with the repair foci.

Julie Wenborn