

Review

Constitutive Histone H2AX Phosphorylation and ATM Activation, the Reporters of DNA Damage by Endogenous Oxidants

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ABSTRACT

DNA in live cells undergoes continuous oxidative damage caused by metabolically generated endogenous as well as external oxidants and oxidant-inducers. The cumulative oxidative DNA damage is considered the key factor in aging and senescence while the effectiveness of anti-aging agents is often assessed by their ability to reduce such damage. Oxidative DNA damage also preconditions cells to neoplastic transformation. Sensitive reporters of DNA damage, particularly the induction of DNA double-strand breaks (DSBs), are activation of ATM, through its phosphorylation on Ser 1981, and phosphorylation of histone H2AX on Ser 139; the phosphorylated form of H2AX has been named γ H2AX. We review the observations that constitutive ATM activation (CAA) and H2AX phosphorylation (CHP) take place in normal cells as well in the cells of tumor lines untreated by exogenous genotoxic agents. We postulate that CAA and CHP, which have been measured by multiparameter cytometry in relation to the cell cycle phase, are triggered by oxidative DNA damage. This review also presents the findings on differences in CAA and CHP in various cell lines as well as on the effects of several agents and growth conditions that modulate the extent of these histone and ATM modifications. Specifically, described are effects of the reactive oxygen species (ROS) scavenger N-acetyl-L-cysteine (NAC), and the glutathione synthetase inhibitor buthionine sulfoximine (BSO) as well as suppression of cell metabolism by growth at higher cell density or in the presence of the glucose antimetabolite 2-deoxy-D-glucose. Collectively, the reviewed data indicate that multiparameter cytometric measurement of the level of CHP and/or CAA allows one to estimate the extent of ongoing oxidative DNA damage and to measure the DNA protective-effects of antioxidants or agents that reduce or amplify generation of endogenous ROS.

DNA DAMAGE BY ENDOGENOUS OXIDANTS

Being continuously exposed to oxidants produced during metabolic activity and to external oxidants or oxidant-inducers, DNA within cells undergoes oxidative damage. Estimates of the extent of endogenous DNA damage vary widely.¹⁻⁵ According to one of the relatively low estimates, during a single cell cycle of average duration (24 h) approximately 5,000 DNA single-strand lesions (SSLs) are generated per nucleus by endogenous reactive oxygen species (ROS).⁵ The SSLs consist of single-strand breaks, apurinic/apyrimidinic sites, oxidation products such as 8-oxoguanine and thymine glycol, and some alkylation products.¹⁻⁵ Approximately 1% of these SSLs become converted to DNA double-strand breaks (DSBs), predominantly at the time of DNA replication, while the remaining 99% are repaired by essentially error-free mechanisms. Thus, on average, about 50 DSBs ("endogenous DSBs") per nucleus (~0.8 DSBs per 10⁸ bp) are generated during a single cell cycle in human cells.⁵ Recombinatorial repair (also known as template-assisted repair or homologous recombination repair) and nonhomologous DNA-end joining (NHEJ) are two major pathways for repair of DSBs. The NHEJ pathway is error-prone, often resulting in deletion of a few base pairs.^{6,7} This leads to accumulation of DNA damage with each sequential cell cycle, which is considered to be the primary cause of cell aging and senescence.^{4,8,9} The cumulative DNA damage also promotes development of preneoplastic changes. Many strategies aimed at slowing down the aging process or preventing cancer are based on protection of DNA from oxidative damage, primarily by scavenging the endogenous oxidants. While approaches to measure the presence of oxidants within the cell have been developed,^{10,11} the possibilities for detecting DSBs generated by endogenous oxidants are limited. DNA damage assessment by the comet methodology¹² is rather cumbersome, lacks the desired sensitivity and cannot provide information on the relationship between DNA damage and the cell cycle phase in which

the damage occurred. The detection of DSBs by the TUNEL assay, while highly useful in identifying DNA fragmentation that occurs during apoptosis,¹³ also is not sensitive enough to detect DNA damage induced by endogenous oxidants. Low sensitivity is also the Achilles heel of other methods developed to measure in situ DNA damage and repair.¹⁴ Methods that measure DNA damage in bulk rather than in situ do not provide information on the response of individual cells, cell population heterogeneity or the relationship of DNA damage to cell cycle phase.

HISTONE H2AX PHOSPHORYLATION AND ATM ACTIVATION, THE MARKERS OF DNA DAMAGE

One of the variants of the nucleosome core histone H2A, histone H2AX undergoes phosphorylation on *Ser* 139 in response to DNA damage, particularly if the damage involves formation of DNA double-strand breaks (DSBs).^{15,16} Its phosphorylation, in nucleosomes located within a megabase domain of DNA on each side of the DSB,¹⁷ is mediated by the PI-3-like kinases ATM-¹⁸ ATR-¹⁹ and/or DNA-dependent protein kinase (DNA-PK).²⁰ The *Ser*-139-phosphorylated H2AX has been named γ H2AX.¹⁴ The presence of γ H2AX manifests in the form of distinct nuclear γ H2AX immunofluorescent (IF) foci.¹⁵⁻¹⁷ Several signaling and repair proteins including the M/R/N complex (Mre11/Rad50/Nbs1), Brca1 and the p53 binding protein 1 (53BP1) colocalize with phosphorylated H2AX at the foci.²¹⁻²⁴ Phosphorylation of H2AX is considered to be a specific reporter of DSBs.^{15-17,25-27} Cytometric detection of phosphorylated H2AX combined with analysis of cellular DNA content provides an assay of DNA damage (e.g., induced by genotoxic agents such as some antitumor drugs) in relation to the cell cycle phase. An example of induction of H2AX phosphorylation in cells treated with DNA topoisomerase I inhibitor topotecan (Tpt) is presented in Figure 1.

H2AX is also phosphorylated in physiological recombinatorial events such as in V(D)J and class-switch recombination during immune system development and also at sites of DSB formation in meiosis.²⁸⁻³¹ Likewise, DSBs generated during DNA fragmentation in apoptotic cells induce phosphorylation of H2AX, the extent of which, however, is much greater compared to its phosphorylation induced by exogenous genotoxic agents.³²⁻³⁴ In fact, the large difference in the degree of H2AX phosphorylation makes it possible, based on the intensity of γ H2AX IF, to discriminate between the apoptotic cells and the cells with primary DSBs generated by DNA damaging agents such as ionizing radiation or by DNA topoisomerase inhibitors.³³⁻³⁵

DNA damage triggers activation of *Ataxia telangiectasia* mutated (ATM) through its phosphorylation on *Ser*1981, and, as noted, H2AX is a substrate of this kinase.^{18,36-40} Whereas H2AX can be phosphorylated by ATR in response to other types of DNA damage, its phosphorylation when mediated by ATM appears to be strongly linked to the induction of DSBs.³⁶⁻⁴⁰ Thus, ATM activation and H2AX phosphorylation observed within the same cells provide strong evidence of the induction of DSBs.³⁷⁻⁴¹ The immunocytochemical detection of γ H2AX concurrent with activated ATM using Abs tagged with a fluorochrome of the same emission wavelength is a very sensitive assay to detect DNA damage that involves formation of DSBs.⁴¹ Figure 1 illustrates the induction of ATM activation concurrent with H2AX phosphorylation, selectively in S-phase cells, by Tpt.⁴¹

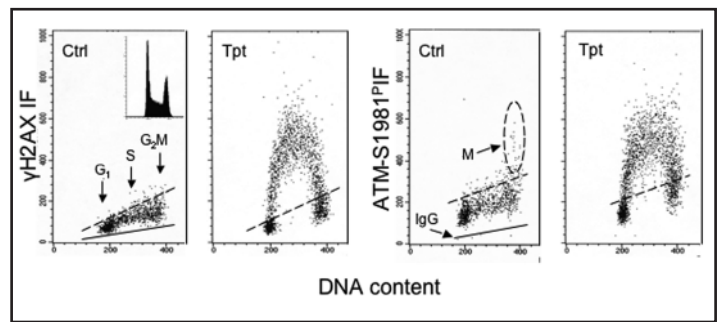


Figure 1. Induction of H2AX phosphorylation and activation of ATM in HL-60 cells by the DNA topoisomerase I inhibitor topotecan (Tpt). Bivariate (cellular IF vs DNA content) distributions showing the selective response of S-phase cells to Tpt-induced DNA damage by histone H2AX *Ser*139 phosphorylation and ATM activation (phosphorylation of *Ser*1981). Untreated (Ctrl) cells or cells exposed to 150 nM Tpt for 2 h were fixed and subjected to immunocytochemical detection of γ H2AX or ATM-S1981^P concurrent with DNA content measurement by flow cytometry.⁴¹ The inset shows the cellular DNA content frequency histogram from untreated culture; no significant changes in the cell cycle distribution was apparent during exposure to Tpt. The solid lines represent the upper level of the fluorescence intensity of the control cells stained with an irrelevant isotype IgG instead of the specific primary Ab. Dashed lines show the upper limit of IF (maximum level for >95% of interphase cells) of the untreated cells. However, mitotic (M) cells from untreated cultures show the presence of activated ATM.⁴³⁻⁴⁷ Note selective response of S-phase cells to the drug.

CONSTITUTIVE HISTONE H2AX PHOSPHORYLATION (CHP) AND ACTIVATION OF ATM (CAA)

Given the above, it could be expected that during unperturbed growth of cells (i.e., in the absence of any exogenous genotoxic agents) there will be a background level of H2AX phosphorylation, and perhaps also activation of ATM, reflecting the cell response to oxidative DNA damage induced by endogenous oxidants that arise from the metabolic activity occurring during cell cycle progression. Indeed, we^{33,43} and others⁴² observed that in untreated, normal cells as well in the cells of various tumor lines, a fraction of the histone H2AX molecules remain phosphorylated. The extent of this constitutive H2AX phosphorylation (CHP) varies depending on the cell type (line) and on cell cycle phase.^{33,43} Figure 2 illustrates CHP variability among 6 different cell lines and in normal human bronchial epithelial cells (NHBE), the latter having the highest level of γ H2AX expression. Cells also constitutively express activated ATM and, as with CHP, the degree of constitutive ATM activation (CAA) differs depending on cell type and cell cycle phase.^{40,43}

It should be stressed, however, that mechanisms of CHP and CAA are different in interphase and in mitotic cells.⁴⁴⁻⁴⁸ While CHP and CAA in interphase cells most likely reflect a response to DNA damage that may involve the presence of DSBs, in mitotic cells these events may be associated with chromatin condensation. For example, the induction of premature chromosomes condensation triggers CHP and CAA even though there is no evidence of DSB formation.⁴⁸ It has been speculated that the changes in chromatin structure during mitosis associated with the induction of torsional stress on the DNA double helix that make it more sensitive to single-strand nucleases⁴⁹ or susceptible to denaturation,^{50,51} may be the signal triggering H2AX phosphorylation and ATM activation.⁴⁸ Furthermore, in mitotic cells, activated ATM is also localized at centrosomes where its function may be associated with the monitoring

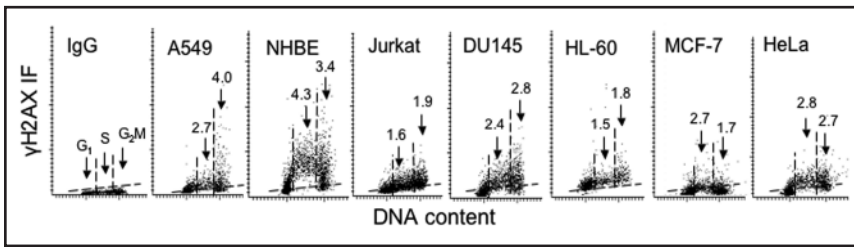


Figure 2. Different levels of CHP in different cell types in relation to cell cycle phase. Bivariate distributions (scatterplots) of cellular DNA content vs γ H2AX IF of human cells from exponentially growing untreated lung adenocarcinoma A549, normal bronchial epithelial (NHBE), T-cell leukemic Jurkat, prostate carcinoma DU145, leukemic HL-60, breast carcinoma MCF-7 and cervical carcinoma HeLa cell cultures measured by laser-scanning cytometry, as described.⁴³ The negative isotype control (IgG; A549 cells) is shown in the panel on the left; the maximal level of IgG IF, below which >97% cells in the IgG panel were located, is marked in each panel with a broken line (other cell types had similarly low IgG IF). The vertical dashed lines show the gates used to separate G_1 , S and G_2 M cell subpopulations to obtain their mean values of γ H2AX IF. The figures above the arrows pointing to S and G_2 M cells indicate the n-fold higher mean expression of γ H2AX of S and G_2 M phase cells, respectively, compared to G_1 cells, whose mean γ H2AX (for each cell type) was normalized, to 1.0.

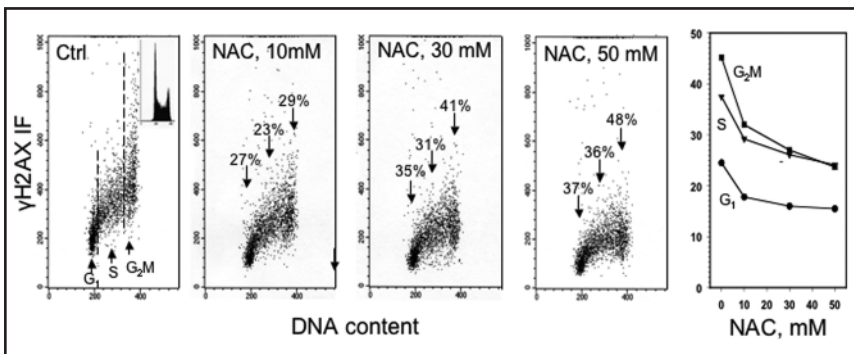


Figure 3. Decrease in CHP in TK6 cells induced by their treatment with different concentration of NAC. The bivariate (DNA content vs γ H2AX IF) distributions illustrate CHP of cells growing in the absence (Ctrl) or presence of 10, 30 or 50 mM NAC, added into cultures for 1 h prior to cell harvesting. The DNA content frequency histogram representative of the cell in these cultures is shown in the inset of the left panel. The percent decrease in mean values of γ H2AX IF of G_1 , S and G_2 M phase cell populations in cultures treated with NAC in relation to the untreated (Ctrl) cells are marked above the arrows pointing out towards the cells in the respective phases of the cycle. The plot of the mean values of γ H2AX IF for G_1 , S and G_2 M cell populations, estimated by gating analysis, as a function of NAC concentration, is shown in the right panel. For details see reference 41.

of mitotic spindle integrity.⁴⁶ Phosphorylation of H2AX at mitosis is also a response to telomere shortening and is associated with induction of cell senescence or apoptosis.⁴⁸

The present review is focused on CHP and CAA in interphase cells and summarizes the findings that provide the linkage suggesting that these events reflect DNA damage by endogenous oxidants. The reviewed data also underscore the fact that multiparameter cytometric analysis of CHP and CAA provides a sensitive and convenient assay for the DNA protective properties of antioxidants and potential anti-aging agents.

THE RELATIONSHIP BETWEEN H2AX PHOSPHORYLATION AND TP53

As mentioned, the presence of the phosphorylated form of H2AX (γ H2AX) has been detected immunocytochemically in a variety of cell types not subjected to treatment with any genotoxic

agent.^{33,42,52} The bivariate distributions of cellular DNA content vs γ H2AX immunofluorescence (IF) revealed CHP in relation to cell cycle phase (Fig. 2). It is quite evident from these distributions that the level of CHP varies markedly depending on the cell type. For example NHBE cells or cells from the A549 or DU145 lines have distinctly higher expression of γ H2AX than the cells from Jurkat, MCF-7 or HL-60 lines. Interestingly, among a variety of cell types analyzed, the cells harboring wt TP53 had generally more elevated levels of CHP compared with the cells that had either mutated TP53 or were null for TP53.⁵² Thus, among the human lymphoblastoid cell lines derived from WIL2 cells, the TK6 cells having wt TP53 showed significantly higher expression of γ H2AX than NH32 cells, the TP53 knock-out derived from TK6, or WTK1, a WIL2-derived line harboring a homozygous mutant of TP53.⁵² The degree of CHP was also low in TP53 null HL-60 cells.⁵² The level of reactive oxygen species detected by the cell's ability to oxidize carboxyl-dichlorodihydrofluorescein diacetate,^{10,11} however, was not significantly different in any of these cell lines, which would suggest that oxidative DNA damage was similar in these lines. The observed higher level of CHP in cells harboring wt TP53, therefore, may indicate that TP53 plays a role in facilitating histone H2AX phosphorylation, the step that mobilizes the DNA repair machinery to the site of DSBs. This function of TP53 is compatible with its role in sensing oxidative DNA damage and facilitating base excision repair,⁵³⁻⁵⁵ and also with its direct and rapid binding to sites of DSBs and interaction with γ H2AX.⁵⁶

SENSITIVITY OF CHP AND CAA TO N-ACETYL-L-CYSTEINE (NAC), AN ROS SCAVENGER

The level of CHP was markedly attenuated in the TK6 cells growing in the presence of NAC (Fig. 3). The effect was NAC concentration-dependent and was already apparent in cell treated with 10 mM concentration of this antioxidant. At 50 mM NAC, the mean γ H2AX IF of G_2 M cells was reduced by 48%, whereas the reduction of γ H2AX IF of G_1 - and S-phase cells was 37 and 36%, respectively. Significant reduction of CHP was also observed in A549 and NHBE cells that were grown in the presence of NAC.⁴³ In contrast, growth of A549 cells in the presence of buthionine sulfoximine (BSO), an inhibitor of glutathione synthetase, for the approximate duration of one cell cycle, led to a distinct rise in the level of CHP.⁴³ The BSO-induced CHP increase was more pronounced for cells in S and G_2 M phase (61–62%) than for G_1 phase cells (30%). All these data, collectively, strongly suggest that the CHP observed in untreated, proliferating cells is a reflection of the ongoing DNA damage caused by endogenous oxidants. Their scavenging by NAC, or elevation as a result of depletion of glutathione by BSO, modulates the extent of CHP accordingly.

The level of CAA was also markedly diminished in the TK6 cells treated with NAC (Fig. 4). As in the case of CHP, the reduction of

CAA was NAC concentration-dependent. At 50 mM NAC, the mean ATM-S1981^P IF of G₁-, S- and G₂- phase cell populations was diminished by 51–53% compared to the untreated cells. It should be noted however, that the ATM-S1981^P IF of mitotic cells essentially was unaffected by NAC.

CHP AND CAA IN CELLS WITH DIFFERENT METABOLIC ACTIVITY

Given that CHP and CAA reflect DNA damage caused by endogenous oxidants generated during metabolic activity it would be expected that the level CHP and CAA may be correlated with the overall rate of cell metabolism. Therefore, it seemed important to determine whether suppression of metabolic activity would lead to a decrease in the levels of CHP and CAA. Towards this end, the levels of CHP and CAA have been assessed in cells that were grown for up to 24 h in the presence of the glucose antimetabolite 2-deoxy-D-glucose (2-DG).⁵⁷ 2-DG competitively inhibits the uptake and utilization of glucose thereby suppressing glycolysis and reducing energy production in mitochondria that should lead to a decrease in production of ROS.⁵⁸⁻⁶⁰ It has been suggested that, similar to caloric restriction or reduced oxygen levels,⁶¹ a nontoxic anti-metabolite might be used as a “caloric restriction mimetic” to lower the extent of DNA damage by oxidative stress and, as a result, expand the life span of cells or organisms.^{58,62} The studies⁵⁷ were carried out on three different human lymphoblastoid cell lines (TK6, NH32 and WTK1), which as mentioned above, differed in their TP53 status.⁵³ A distinct attenuation of CHP and CAA in cells growing in the presence of 2-DG was observed for all three lines; a significant decrease was observed as early as 4 h following exposure to 2-DG.⁵⁸ This data provided further support for the notion that CHP is a reporter of oxidative DNA damage caused by ROS during the metabolic processes fueled by glycolysis when cells progress through the cell cycle. Additionally, the data suggested that ATM either alone or in concert with other PI-3-like kinase(s), mediated CHP.⁵⁷ Figure 5 shows the data selected from this report⁵⁷ and illustrates the effect of exposure of WTK1 cells to 2-DG for 24 h on the level of CHP and CAA. It is quite evident that the cells growing in the presence of 2-DG had both markedly diminished CHP and CAA. The reduction of CAA was more pronounced (45–51%) than the decrease of CHP (27–43%). These observations are consistent with our recent findings that growth in the presence of 2-DG reduced the level of ROS as reflected by a decrease in cell ability to oxidize carboxyl-dichlorodihydrofluorescein diacetate (unpublished).

Cell growth at higher cell density in cultures reduces their proliferation and also attenuates their metabolic activity as reflected by retention of rhodamine 123 a probe of mitochondrial mass and potential, as well as by reduced rates of RNA and protein synthesis.⁶³⁻⁶⁵ Figure 6 presents the level CHP and CAA in TK6 cells

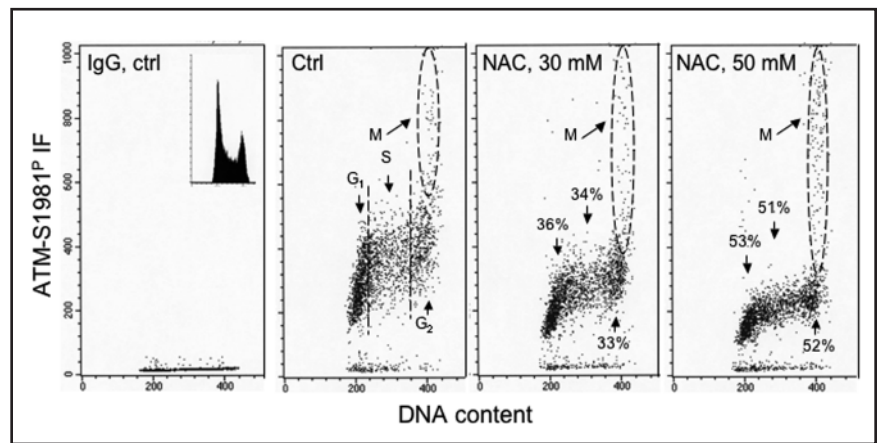


Figure 4. Decrease in CAA in TK6 cells treated with different concentrations of NAC. The bivariate (DNA content vs ATM-S1981^P IF) distributions illustrate CAA of cells growing in the absence (Ctrl) or presence of 30 or 50 mM NAC included into cultures for 1 h prior to cell harvesting. The left panel shows the IgG isotype (negative) control; the DNA content frequency histogram representative of the cell in these cultures is shown in the inset of this panel. Mitotic cells (M) have a high level of ATM-S1981^P IF⁴³⁻⁴⁷ and are marked by the oval dashed-line boundaries. The percent decrease in mean values of CAA of G₁, S and G₂ (M cells excluded) cell populations in cultures treated with NAC in relation to the untreated (Ctrl) cells are marked above the arrows pointing out towards the cells in the respective phases of the cycle.

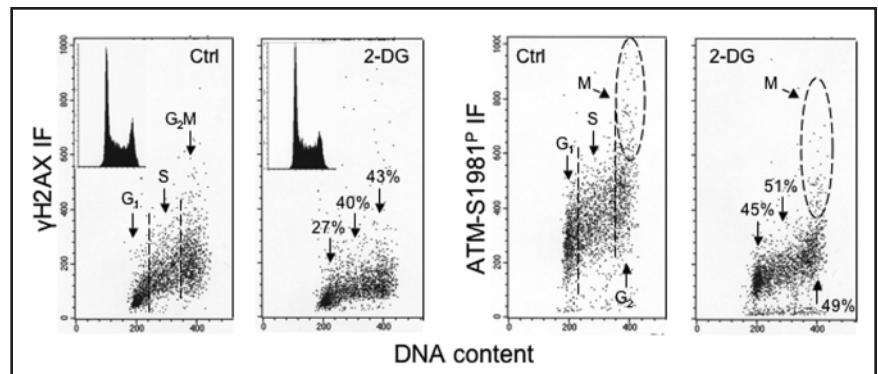


Figure 5. Decrease in the level of CHP and CAA in human lymphoblastoid NH32 cells grown in the presence of 2-deoxy-D-glucose (2-DG). The bivariate (DNA content vs γ H2AX, or vs ATM-S1981^P) distributions of untreated cells (Ctrl), and cells growing in the presence of 5 mM 2-DG for 24 h. The DNA content frequency histograms, representative of the untreated and treated cells, are shown in the respective insets. Mitotic cells (M) express high level ATM-S1981^P IF which appears unrelated to endogenous DNA damage-induced CAA.⁴²⁻⁴⁵ The percent decrease in mean values of γ H2AX IF or ATM-S1981^P IF of G₁, S, and G₂M phase cell populations (only G₂ phase cells in the case of ATM-S1981^P) in cultures treated with 2-DG in relation to untreated (Ctrl) cells is listed above or below each arrow pointing towards the cells in the respective phases of the cycle.

growing at densities 2×10^5 , 10^6 and 2×10^6 cells/ml, respectively. These data demonstrate that CHP and CAA levels were distinctly reduced in cells maintained at higher densities. As in the case of cell growth in the presence of 2-DG, the reduction was more pronounced for CAA (40–46%) than for CHP (17–27%). It should be stressed that the observed reduction of CHP and CAA induced by 2-DG⁵⁷ or by growth at higher densities was not accompanied by any cytotoxic effects. In the case of growth at higher densities a moderate decrease in the proportion of S and G₂M phase cells was apparent (Fig. 6, see the insets), consistent with the reduced rate of proliferation at higher cell densities.⁶⁵

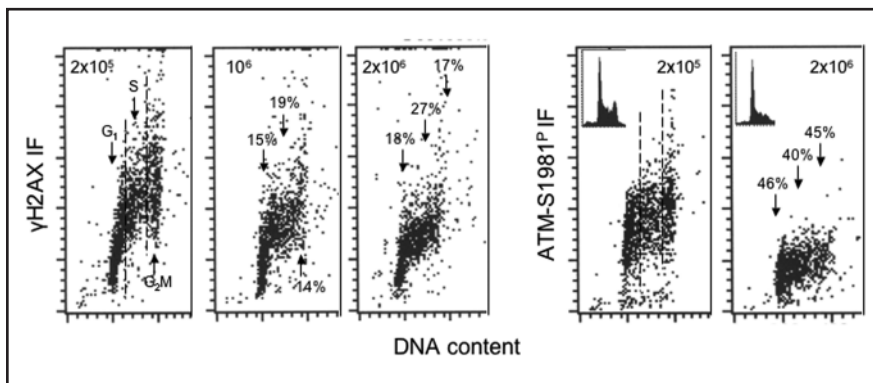


Figure 6. Decreased levels of CHP and CAA in TK6 cells growing at higher cell densities. Human lymphoblastoid TK6 cells were seeded in cultures at different densities and were harvested when the densities approached 2×10^5 , 10^6 or 2×10^6 cells/ml. The bivariate (DNA content vs γ H2AX, or vs ATM-S1981^P) distributions illustrate a decline in CHP and CAA of cells growing at higher densities. Insets in the right panels show DNA content histograms in these cultures. The percent decrease in mean values of γ H2AX IF or ATM-S1981^P IF of G₁, S and G₂M cell populations in cultures at 10^6 or 2×10^6 cells/ml with respect to the cells growing at density 2×10^5 cells/ml is shown above each arrow pointing towards the cells in the respective phase of the cycle.

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