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Activation of self-renewal and meiotic genes in tumour cell lines resistant to genotoxic treatment

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Abstract

Previously, resistant re-growth of tumours after genotoxic treatments was shown to be associated with cancer stem cells, however the induced reversible polyploidy and meiotic genes were also found as involved. In the present work, a possible link between both phenomena, depending on TP53 status was addressed on lymphoma and HeLa cell lines. For the first time, the upregulation of the master stemness transcription factors OCT4, NANOG and SOX2 induced by overcoming the tetraploidy barrier by the DNA damaged TP53 mutant but not wild-type tumour cells, as well as expression of the key meiotic cohesin REC8 in somatic tumour cell lines treated with ionizing irradiation or topoisomerase II inhibitor etoposide were found and cytologically characterised. In addition, the role of autophagy in this process was found. Collectively, these observations characterise survival strategy of TP53-mutant tumour cells recovering after genotoxic treatments through nuclear reprogramming to the embryonal-like state.

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Introduction

Cancer is responsible for each third death in developed countries. In Latvia cancer desease is the second most common cause of death, causing about 20% of all deaths. One of the major problems in cancer therapy is the formation of the secondary resistant tumour cells after initially successful genotoxic treatments, such as chemo- and radiotherapy, which cause relapse of the disease and metastases in up to 50% of cancer cases, which subsequently kill the patients. The biological nature of this resistance is not yet clear.

Recently tumour cell resistance has been attributed to the presence of cancer stem cells (CSC) in the tumour population (Jordan *et al.,* 2006) and to the intrinsic properties of cancer stem cells, most notably their enhanced expression of ABC drug efflux pumps, augmented DNA repair capacity, adaptation to hypoxia, and inherent resistance to apoptosis (Baumann *et al.,* 2008). Alternatively, the hypothesis was proposed that the differentiating and proliferating tumour cells derived from progenitors can reactivate stemness and self-renewal-capability (Blagosklonny 2007). The previous study in our laboratory showed that recovery of resistant tumour cells after genotoxins is associated in TP53 mutant tumour cells with induced polyploidy and its reversal (Erenpreisa *et al.,* 2000; 2005, 2008). The hypothesis of the cancer cell 'life cycle' postulating cyclic character of this process was suggested (Erenpreisa and Cragg 2007). In the present study, the elaborated models of the previous research on irradiated lymphoma cell lines were used to study the possible relationship between reversible polyploidy and reprogramming to the germline-like state.

The aim and tasks of the study:

The aim of this study was to investigate the expression of the key self-renewal and meiotic genes in somatic tumour cell lines and its dependence on the different status of the *TP53* after DNA damage induced polyploidization. Several tasks were set to achieve these goals:

- To study the possible role of ectopically induced meiotic cohesin REC8 in reversible polyploidisation of tumour cells.
- To study expression of the key pluripotency and self-renewal genes (OCT4, NANOG, SOX2) after DNA damage in association with induced reversible polyploidy
    - in wild type TP53 tumour cell line (TK6 lymphoblastoma, parental to WI-L2-NS)
    - in mutated TP53 tumour cell lines (Burkitt' lymphoma Namalwa and lymphoblastoma WI-L2-NS).
- To study the possible contribution of the chromatin autophagy in reversible polyploidy and its relation to self-renewal.

1. Literature overview

1.1 Cancer genome and biology

The hallmarks of cancer characterising its essential biological features were defined by Hanahan and Weinberg in 2011. They have formulated them as „ six biological capabilities acquired during the multistep development of human tumors". They include sustained (constitutive) proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Conceptual progress in the last decade has added two emerging hallmarks of potential generality to this list - reprogramming of energy metabolism and evading immune destruction (Hanahan and Weinberg 2011).

Two main models of cancer development and progression to metastatic disease are established: the clonal evolution and cancer stem cell models. The clonal evolution theory suggests that cancer development is based on accumulation of random mutations and natural selection in response to microenviromental pressure what generate clonal growth in given cell, dictating the phenotype of the tumor (Nowell *et al.,* 1976).
In addition, cancer stem cell (CSC) model suggests that cancer cells with similar genetic backgrounds can be hierarchically organized and CSCs are thought to have tumor-initiating and metastatic ability (Bonnet and Dick 1997). However, evolving evidence shows that considerable plasticity exists between the non-CSC and CSC compartments, such that non-CSCs can re-acquire a CSC phenotype (Marjanovic *et al.,* 2013).

It fits, in fact, the most old embryological theory of cancer, which postulated that cell embryonalisation is the biological essence of carcinogenesis (Erenpreiss 1993).

There is an evidence that at least 350 (1.6%) of the 22,000 protein-coding genes in the human genome contribute to cancer development showing recurrent somatic mutations (Stratton *et al.,* 2009). In some cases, over 1,000 mutations were registered. It is thought that the 'driver'mutations give growth advantage and they are positively selected during the envolvement of the cancer. While others are 'passengers' mutations that do not confer growth advantage, but happened to be present in an ancestor of the cancer cell when it acquired one of its drivers. It is thought that most cancers carry more than one driver and that the number varies between cancer types. It has been suggested that common adult epithelial cancers such as breast, colorectal and prostate require 5–7 rate-limiting events, possibly equating to drivers, whereas cancers of the haematological system may require fewer mutations.

Cancer-critical genes are grouped into two broad classes: 1) proto-oncogenes (their overactivation or overexpression can drive a cell toward cancer); 2) tumor suppressor genes (loss-of-function mutation can contribute to cancer).

Proto-oncogenes code for proteins that help to regulate cell growth and differentiation (MYC), are often involved in signal transduction (RAS, WNT) and execution of mitogenic signals (ERK). Tumor-suppressor genes encoded proteins are involved in the regulation of the cell cycle and promoting apoptosis. Mutant tumor suppressors alleles are usually recessive whereas mutant oncogene alleles are typically dominant. There are tumor-suppressor genes that are exceptions and exhibit haploinsufficiency, for example TP53 (Kurzhals *et al.,* 2011). Epigenetic modifications like changes in CpG island methylation patterns, histone modifications and MicroRNA gene silencing are involved in silencing of tumor suppressor genes and activation of oncogenes.

The defects affecting various components of the DNA-maintenance machinery—often referred to as the ''care-takers'' of the genome (Kinzler and Vogelstein, 1997)— behave much like tumor suppressor genes, because their functions can be lost during the course of

tumor progression, with such losses being achieved either through inactivating mutations or via epigenetic repression. This includes genes whose products are involved in (1) detecting DNA damage and activating the repair machinery, (2) directly repairing damaged DNA, and (3) inactivating or intercepting mutagenic molecules before they have damaged the DNA (Hanahan and Weinberg 2011).

The loss of telomeric DNA, in many tumors generates genomic instability and associated amplification and deletion of chromosomal segments (Artandi and DePinho, 2010).

1.2 The stem-like properties of cancer cells determine their resistance to anti-cancer treatment

In recent years the hypothesis of cancer stem cells has been actualised - presuming that only a small portion of cancer cells are able to maintain renewal of cancer. In literature these cells have been called cancer stem cells (CSC), tumor initiating cells (TIC), stem-like cancer cells and also stemloids – the close progenitors of stem cells (Blagosklonny 2007). CSCs are defined operationally through their ability to efficiently form new tumors upon inoculation into recipient host mice (Cho and Clarke, 2008). This functional definition is often complemented by including the expression in CSCs of markers that are also expressed by the normal stem cells in the tissue-of-origin (Al-Hajj *et al.,* 2003). Fractionation of cancer cells on the basis of displayed cell-surface markers has yielded subpopulations of neoplastic cells with a greatly enhanced ability, relative to the corresponding majority populations, to originate new tumors upon implantation in immunodeficient mice. These tumor-initiating cells share transcriptional profiles with certain normal tissue stem cell populations, indicating to their origin from adult (tissues-located) stem cells (Hanahan and Weinberg 2011). Several putative cancer stem cell markers has been used: (1) cell surface glycoproteins including CD133, CD44, CD24, and CD166, (2) aldehyde dehydrogenase 1 (ALDH1) – enzyme that is responsible for the oxidation of aldehydes and thus participates in cellular detoxification, differentiation, and drug resistance (Jiang *et al.,* 2009), (3) expression of ABC efflux pumps (4) markers for signaling pathways such as Notch, Wnt and Hedgehog.

The origins of CSCs within a solid tumor are unclear. There are two hypothesis:

1. Subpopulation of CSC arise from the oncogenically mutated adult stem cells (Baumann *et al.,* 2008).
2. Stemness status may be induced in any malignant (mutant) progenitor or differentiated cells by stress conditions (Blagosklonny 2006; 2007).

Increasing evidence in a variety of tumor types suggests that cells with properties of CSCs are more resistant to various commonly used chemotherapeutic treatments (Singh and Settleman, 2010). In addition it is shown the enrichment with CSC in tumour cell population after irradiation, which was explained by their positive selection after treatment (Phillips *et al*., 2006). Tumour cell resistance to anticancer treatment has been attributed to the properties of these cells to enhance expression of ABC drug efflux pumps, increase DNA repair capacity and resistance to apoptosis (Baumann *et al.,* 2008). It is suggested also, that differentiated and proliferating tumour cells derived from progenitors can reactivate stemness and self-renewal-capability (Blagosklonny 2007).

1.3 Induced reversible polyploidy of tumour cells serves for escape from genotoxic treatment.

1.3.1 Polyploidy in normal cells

Polyploidisation, the addition of a complete set of chromosomes to the genome, represents one of the most dramatic mutations known to occur (Otto 2007).

Mammals are diploid, except the red vizcacha rat (Tympanoctomys barrerae) and its close relatives, which are tetraploid (Gallardo *et al.* 1999). In other mammals, tetraploidy causes early lethality and spontaneous abortion or resorption of the embryo (Kaufman 1991). Although nearly all mammalian species are diploid, the conversion from diploidy to polyploidy is part of normal development and differentiation in several specialized cell types (Davoli and Lange 2011). Placental trophoblast cells, megakaryocytes, hepatocytes, and cardiomyocytes can develop a certain degree of polyploidy during their normal lifespan.

The benefits of programmed polyploidy are not fully understood. First of all, polyploidy provides increased protein amount due to gene dosage effect. Polyploidy results in a decreased surface-to-volume ratio for both the whole cell and the nucleus, thereby minimizing membrane requirements but also lowering the efficiency of transport (Comai 2005), increases the metabolic capacity of a tissue possibly by funneling energy toward gene duplication and protein synthesis instead of cell division and membrane synthesis (Davoli and Lange 2011, Lee *et al.,* 2009). Recently, Duncan *et al.* (2010) proposed that tetraploidization of hepatocytes has the benefit of generating genetic variability. During liver regeneration following damage, tetraploid hepatocytes can undergo a reductive mitosis to create aneuploid daughter cells with a near-diploid chromosome number (Duncan *et al.,* 2010).

In several tissues, tetraploid or octoploid cells appear with aging and under pathological conditions. It was found, that the incidence of polyploidy increases with age in hepatocytes and vascular smooth muscle cells (Celton-Morizur *et al.,* 2010, Hixon and Gualberto 2003), and in vitro human fibroblasts and endothelial cells become tetraploid with replicative aging (Walen 2006; Davoli and Lange 2011). Human cardyomyocytes increase degree of polyploidy upon excessive load and after infarctions and can reduce polyploidy after relief of the load (Rivello *et al.,* 2001).

1.3.2 Induced reversible polyploidy in tumour cells

Most human solid tumors are aneuploid. Karyotypic analysis of cancer cell lines has revealed a wide range of chromosome numbers ranging from hypodiploid to hypertetraploid (Davoli and Lange 2011). Stable aneuploidy is often characteristic for connective and epithelial tumours, for example HeLa cell line has para-triploid genome.

In malignant tumour cells endopolyploidy can be induced by genotoxic stress. Polyploidisation increases the efficiency of DNA double strand breaks repair in additional rounds coupled to re-replication (Ivanov *et al.,* 2003). Although the induced polyploid cells in tumours are unstable and often exposed to death, the evidence has accumulated on reversibility of induced polyploidy. It was shown that a small part of polyploid tumour cells is able to reduce ploidy and form paradiploid clones (Illidge *et al.,* 2000, Sundaram *et al.,* 2004, Puig *et al.,* 2008, Vitale *et al.,* 2010). Evidence to date includes cell sorting experiments in which FACS-isolated endopolyploid cells, produced by both ionising radiation and cytotoxic drugs (cisplatin, nocodazole, doxorubicin), Furthermore, time-lapse imaging has been used to observe polyploidy giant cells undergoing genome reduction to give rise to viable diploid cells (Puig *et al.,* 2008, Ianzini *et al.,* 2009). In addition consecutive endopolyploidisation and

depolyploidisation was suggested to increase tumour heterogeneity, giving rise to resistence and tumour progression (Puig *et al.*, 2008).

Three distinct causes of tetraploidization in cancer have been proposed: (1) cell-to-cell fusion, leading to multinucleated cells that are arrested in the G1 phase of the cell cycle; (2) endoreplication, during which cells enter two consecutive rounds of DNA replication that are not separated by mitosis; or (3) aborted mitosis, during which cells with duplicated chromosomes fail to undergo either nuclear division (karyokinesis) and reset interphase ”mitotic slippage” or fail to undergo cytokinesis and form multi-nucleated polyploid cells. Just the aborted mitosis was most often observed after genotoxic treatments of tumour cells (Ivanov *et al,* 2003; Erenpreisa *et al.,* 2005, 2008). Currently the significance of each kind of cell division (for example bi-polar and/or multi-polar) or/and fusion mechanism in clonogenic survival after DNA damage is unclear.

The reversible polyploidisation observed in tumour cells after DNA-damage is a complex process that gives clonogenic escape only 10^4-10^6 of the treated cells (Puig *et al.,* 2008). Polyploidisation occurs in 10-40% of the cells, reaching a peak on day 5 after genotoxic treatment, with ploidy numbers up to 32n. After extensive cell death only 10-20% of the polyploid giant cells remain alive, some of which undergo process of de-polyploidisation leading to the establishment of the mitotically cycling survivors from day 7-14 after DNA damage. Re-treatment of the cells after recovering causes the same process again.(Illige *et al.,* 2000).

Somatic polyploidy (endopolyploidy) can be reversible and irreversible. Irreversible polyploidy occurs during development through re-replication in the absence of mitosis, but reversible polyploidy of mammalian tumour cells, mammalian hepatocytes, cardiomyocytes and megakaryocytes (Zybina and Zybina 2011) occurs mostly through aborted polyploidising mitoses and is less extensive, forming mainly 4n-8n progeny and typically not exceeding 32n. Polyploidy is suggested to be as a link between cellular senescence, cancer development and possible cancer renewal after treatment (Moseniak and Sikora 2010; Erenpreisa and Cragg 2013).

Stable tetraploid cell lines showed enhanced viability in comparison to parental diploid cell lines and this effect surpassed that of the absence of TP53 function: tetraploid HCT116GH expressed lower level of p53 and p21 than diploid HCT116 (Park *et al.,* 2011).

1.3.3 The tumour suppressor TP53 and its role in the formation of endopolyploidy cells

DNA and spindle damage induce polyploidy in tumour cells, particularly when TP53 function is absent or dysregulated. This was also confirmed in studies of our laboratory (Illidge *et al.,* 2000; Figure 1).

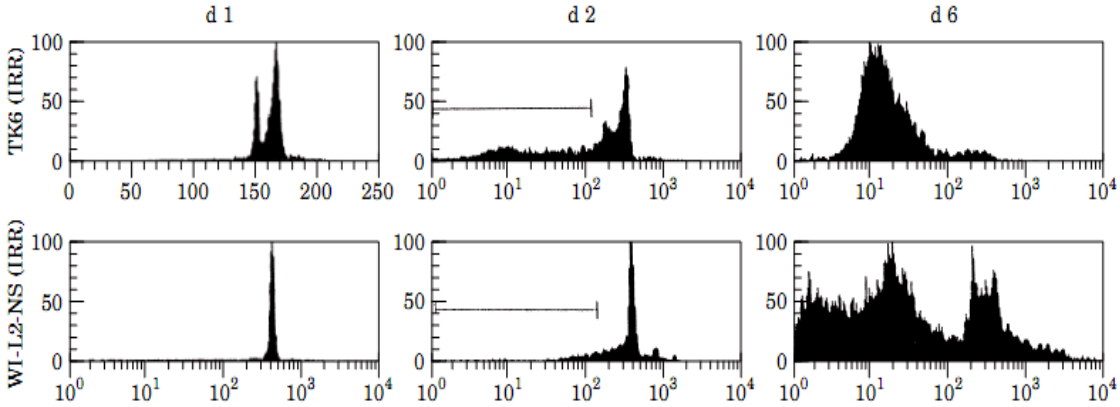

Figure 1. DNA histograms shows the differences in response of TP53 wild-type (TK6) and TP53 mutant (W1-L2-NS) cells to 10 Gy irradiation. The WI-L2-NS cells undergo profound G2-M arrest,

delayed apoptosis and extensive polyploidy, whereas the TK6 cells demonstrate partial G1 arrest, rapid apoptosis and lack a significant polyploid cell response after irradiation, shown over a six day period. Adapted from Illidge *et al.*, 2000.

The p53 tumour suppressor is induced by a variety of stresses through transcriptional, post transcriptional and post-translational mechanisms and it is a master regulator in different biological activities (Menendez *et al.*, 2009). p53 functions include - growth arrest by holding the cell cycle at the G_1/S, activation of DNA repair (Smith *et al.*, 2000), regulation of apoptosis, and cell senescence. p53 has also autophagy-modulatory functions, while nuclear p53 stimulates autophagy by transactivating several pro-autophagic genes, cytoplasmic p53 inhibits the autophagic flow (Maiuri *et al.*, 2010). p53 can also act in mediation of cellular senescence (Moseniak and Sikora 2010), although the data on this function are controversial (Demidenko *et al.*, 2010).

The TP53 tumour suppressor gene is often referred to as the "guardian of the genome", because of its significant role in maintenance of the genome integrity (Kastan 2007). Mutation in TP53 gene, that alters transcriptional activity, is a characteristic feature for approximately 50% of all cancers, while in many others its fuction is compromised by different other means, f.ex. amplifications of its inhibitor MDM2 (Kastan 2007, Menendez *et al.*, 2009).

Mitotic catastrophe and its bypass with the formation of polyploidy cells as a response to genomic insult is a phenomenon of TP53-mutant and -nonfunctional cells (Erenpreisa and Cragg, 2007; Vakifahmetoglu *et al.*, 2008). Cells, that lack functional TP53 can enter metaphase and undergo mitotic slippage, re-enter the tetraploidy cell cycle cells and initiate another round of DNA replication. The p53 protein is involved in G2 DNA damage checkpoint and in inhibition of endopolyploidy (Lanni and Jacks 1998, Aylon and Oren 2011). Therefore, in TP53 mutated tumour cells in response to genotoxic stress, failure to arrest at G1 and subsequent mitotic catastrophe can lead to uncoupling of the spindle checkpoint from apoptosis, restitution of interphase and formation of tetraploid cells. In addition, the absence of TP53 function has been shown to be permissive for induction of reversible polyploidy, survival and mulitpolar division of tetraploid cells, which can result in aneuploidy (Vitale *et al.*, 2010; 2011).

1.3.4 Cancer cell 'life-cycle' hypothesis

The previous study in our laboratory showed that recovery of resistant tumour cells after DNA damage is associated in TP53 mutant tumour cells with the induced reversible polyploidy (Erenpreisa *et al.*, 2000; 2005, 2008). The hypothesis of the cancer cell 'life cycle' presuming cyclic activation of the germline-like properties through the poliploidy induction and its reverse was put forward. It was suggested that tumour cells are capable to slip from normal mitotic into ilicit ploidy cycle allowing resque of survival stem-cell-like descendants after DNA damage. (Figure 2, Erenpreisa Cragg 2007).

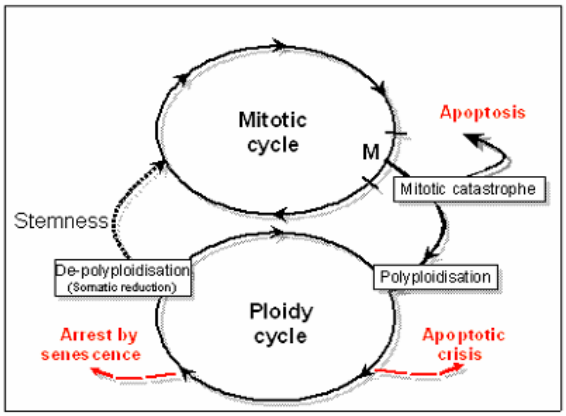

Figure 2. Schematic of a cancer cell life-cycle showing the reciprocal link between the mitotic cycle and the ploidy cycle. Adapted from Erenpreisa and Cragg 2007.

From the point of this hypothesis, the expression of the genes associated with the germline activation in somatic tumour cells can be anticipated.

1.4 Ectopic expression of meiotic genes induced in tumour cells by DNA and spindle damage

Meiosis is a special type of cell division necessary for reproduction and transfer of immortality in the generations of eukaryotes. Its particular function is to provide genetic variation and the sexual process by halving the chromosome number, which is again doubled by fertilisation. Meiosis (after prophase) is driven by MOS kinase (Figure 3) induced in females by progesteron.

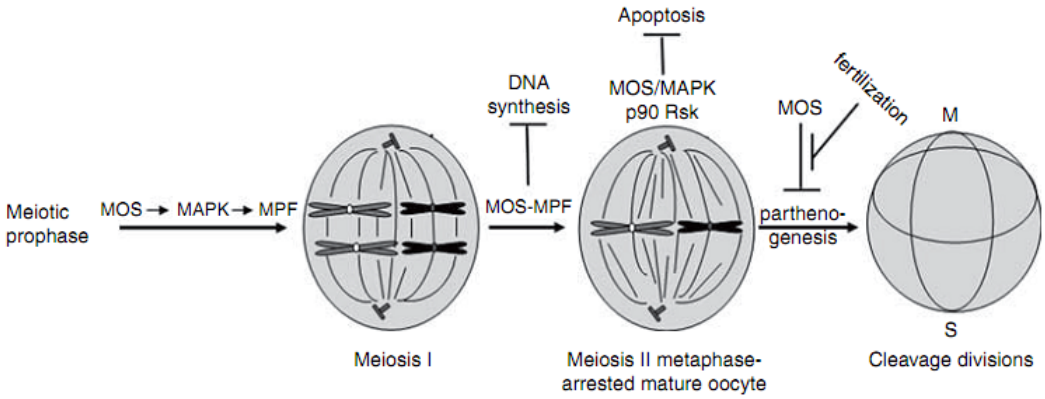

Figure 3. Scheme indicating the participation of MOS in the principal steps of female meiosis: In meiosis I, MOS directly phosphorylates microtubules and organizes the spindle, regulating the contraction and favoring bipolar separation of homologous chromosomes; in interkinesis, MOS prevents DNA synthesis; in meiosis II, MOS activates the spindle checkpoint (in co-operation with cytostatic EMI proteins) and uncouples it from apoptosis. Arrest in metaphase II prevents parthenogenesis of the mature oocyte until fertilization. Adapted from Erenpreisa and Cragg 2010.

Interestingly, aberrant expression of MOS was reported in lung cancer (Gorgoulis *et al.,* 2001) and high levels of Mos were found in nuclei of ETC (Erenpreisa *et al.,* 2005).

The two coupled prerequisites are necessary to execute proper meiotic divisions: (1) Sister chromatid cohesion and homologous chromosome pairing, (2) recombination between homologous chromosomes.

One of the major protein invoved in the both processes is the meiotic cohesin REC8. REC8 belongs to the cohesin protein complex, which participiates in correct chromosome disjunction and homologous recombination in the mitotic and meiotic division cycle (Nasmyth 2001). REC8 provides the cohesion between sister chromatids and particularly centromeres preventing separation until anaphase II (Martin *et al.,* 2009). Together with Shugoshin 1 (SGOL1/SGO1), its function is to promote the reductional cell division in meiosis (Watanabe and Kitajima, 2005).

REC8 is released from chromosome arms and centromeres stepwise, during the two meiotic divisions. Due to SGO1 cohesion and its stabilisation function of REC8-mediated cohesion at sister centromeres, the sister chromatids remain cohesed in the first meiotic division (Brar *et al.,* 2006). The meiotic pairing of homologs (four threads) is dependent on the formation of physiological DSBs by the SPO11 transesterase and their subsequent repair by homologous recombination (Lichten, 2001) which involves, also the meiosis-specific

recombinase DMC1 (Sehorn and Sung, 2004). DMC1 is acting as a heterodimer with RAD51 and the both belong to the conserved family of the prokaryotic recombinase RecA. Proper pairing and recombination between homologous chromosomes and resolution of chiasmata is a condition for the proper reductional division.
The important feature of the chromosome reduction in meiosis is omission of the S-phase between two meiotic divisions, resulting in reduced number of the chromosomes.
It was found that meiosis-specific genes are expressed in the ETCs of the DNA-damaged tumour cells (Kalejs *et al.,* 2006). Ianzini et al showed activation of such meiosis-specific genes as SYCP1, SYCP2, SYCP3, DMC1, SPO11, STAG3, MOS, and also REC8 (Figure 4) in the human tumour cell lines following radiation-induced mitotic catastrophe (Ianzini *et al.,* 2009).

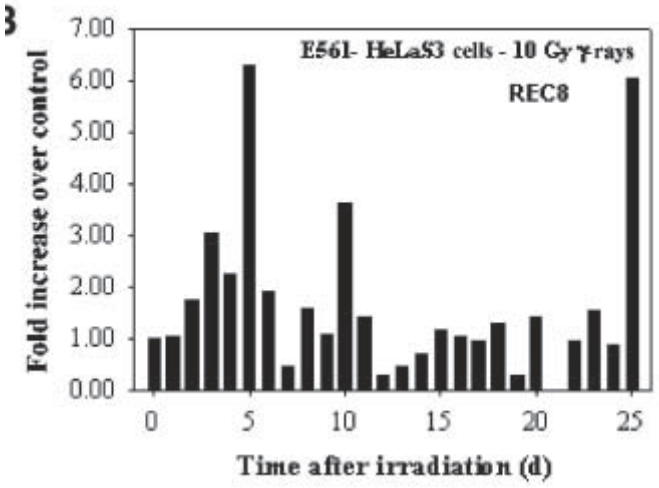

Figure 4. Rec8 mRNA expression in human tumor HeLa S3 cell line after irradiation increases as high as 5-fold by day 5 postirradiation. Adapted from Ianzini 2009.

1.5 Embryonal stem cell genes and their expression in tumours

The embryonal stem cell transcription network which tightly links self-renewal with pluripotency (the capability to produce all three germ layers) is regulated by three master genes OCT4, NANOG and SOX2 (Chambers and Smith, 2004). These three factors occupy 3%, 9% and 7% of the promoter regions of approximately 18,000 genes in human ES cells and synergistically regulate transcription of their targets (Boyer *et al.,* 2005). Pluripotent stem cells derived from preimplantation embryos, primordial germ cells, embryonal carcinoma (teratocarcinoma) or artificially induced from somatic cells (iPSC) by transfection of Yamanaka transcription factors OCT4, SOX2, KLF4, c-MYC (Takahashi and Yamanaka 2006) are unique in undergoing prolonged symmetrical self-renewal in culture. All of these pluripotent cell-types are capable of supporting embryonal development and express key embryonal stem cell (ESC) genes (Chambers and Smith, 2004). The pluripotency network has been found expressed in many types of adult stem cell (Lengner *et al.,* 2007) and in malignant somatic tumours correlating there with poor clinical outcome (Ben-Porath *et al.,* 2008, Saigusa *et al.,* 2009, Ge *et al.,* 2010).

1.5.1 POU5F1 (OCT4)

The most important transcription factor (‘the gatekeeper’ of embryonal development), which is required to maintain an undifferentiated state (self-renewal) and pluripotency of human

ECS is OCT4 (POU5F1) – a member of the POU family of homeodomain transcription factors. The protein level of OCT4 needs to be precisely controlled in ES cells to maintain self-renewal, because even a two fold increase or decrease of the OCT4 protein induces differentiation (Niwa *et al.,* 2000). In mESCs differentiation-independent fluctuation of OCT4 level was found, showing the highest levels of OCT4 occurred in G2 phase (Sustackova *et al.,* 2011).

Three splicing variants of OCT4 gene have been hitherto described with OCT4A the classical form active in transactivation, OCT4 B (POU5F1)_B a cytoplasmic isoform with possible function in cell stress of its alternatively translated variant (Wang *et al.,* 2009) and the recently discovered OCT4 B1 (POU5F1)_B1 splicing isoform (Atlasi *et al.,* 2008) a potential additional marker of stemness (Papamichos *et al.,* 2009). Of seven OCT4 pseudogenes, the 1st (POU5F1B) has been recently confirmed as a transcriptional activator similar to POU5F1 (Panagopoulos *et al.,* 2008), while the 3rd and 4th pseudogenes also have considerable open reading frames with transactivation potential. Recent data shows, that pseudogene POU5F1B is located side by side with MYC proto-oncogene on 8q24 and was frequently involved in MYC amplicons (Hayashi *et al.,* 2012). In addition, a translational isoform of Oct4B-265 is involved in early stress response (Gao *et al.,* 2012), and OCT4 was found to be recruited to the DNA damage sites (Bartova *et al*., 2011), thus participating in the DNA damage response in ESC cells, which is likely TP53-dependent (Jackson *et al.,* 2013).
Knock-down of OCT4 expression in LC-CD133+ cells (from lung cancer patient tissue samples and cell lines) can significantly inhibit the abilities of tumour invasion and colony formation, and increase apoptosis, demonstrating that OCT4 expression plays a crucial role in maintaining the self-renewing, cancer stem-like, and chemoradioresistant properties of LC-CD133+ cells (Chen *et al.,* 2008). Wang *et al.,* (2010) also found that OCT4 can mediate chemoresistance through a potential OCT4-AKT-ABCG2 pathway.

1.5.2 NANOG

Transcriptional regulation of the homeodomain transcription factor nanog1 occurs in the NANOG locus through cooperative interaction with the OCT4:SOX2 heterodimer (Rodda *et al.,* 2005). In turn, nanog1 is a central player in this transcriptional network, which serves to maintain OCT4 levels, sustains pluripotency, facilitates multi-drug resistance, and promotes cytokine independent self-renewal (Chambers *et al.,* 2007). At least eleven pseudogenes of nanog (Booth and Holland, 2004) exist although their expression and function in human tumours are little explored. Intriguingly, at least one of them – a retrogene of nanog1 nanogp8 contains an intact open reading frame that could potentially encode a protein similar to NANOG1 (Palla *et al.,* 2013).

Nanogp8 was shown to be expressed in human cancers (Zhang *et al.,* 2006; Ischiguro *et al.,* 2012) posessing the pluripotency properties similar to the parental and loss-of-function analysis shows inhibition of tumour development (Jeter *et al.,* 2009). Contrary to OCT4, which can be induced through activation of TP53 by DNA damage (Jackson *et al.,* 2013), Nanog1 and TP53 are antagonists: Nanog promoter is directly suppressed by activated TP53 (Lin *et al.,* 2005). The upregulation of NANOG, together with p53 depletion, was significantly associated with clinical late stage of human hepatocellular carcinoma (Wang *et al.,* 2013).

1.5.3 SOX2

SOX2 (SRY (sex determining region Y)-box 2), in conjunction with OCT4, stimulates its own transcription as well as the expression of a growing list of targeted genes. The essential function of SOX2 is to stabilise ESC and neural stem cells into a pluripotent state by maintaining the requisite level of OCT4 (Boyer *et al.,* 2005). SOX2 appears to function as a

molecular rheostat that controls the expression of a critical set of embryonic genes, as well as the self-renewal and differentiation of ESC (Kopp *et al.,* 2008). During differentiation of ESC, the mitotic kinase inhibitor p27kip1 directly suppresses the promoter of SOX2 (Li *et al.,* 2012).

Human fibroblasts (IMR90) can be reprogrammed into induced pluripotent stem (iPS) cells by transfection of Yamanaka combinations of transcription factors such as OCT4, SOX2, KLF4, and c-MYC (OSKM) (Takahashi and Yamanaka 2006). However, the crop of the iPSC is very small, while senescence in the majority of cells senescence is induced, by upregulating p53, p16INK4a, and p21CIP1 (Banito *et al.,* 2009).

1.6 DNA damage response and the G2M checkpoint

The DNA damage response (DDR) is a complex process that involves proteins, comprising DNA damage recognition, signal transduction, transcriptional regulation, cell cycle control and DNA repair. The loss of certain DDR components can lead to an increased susceptibility to cancer due to the ensuing genomic instability and the subsequent increased mutability of the genes required for cellular replication and division. The DDR is also involved in the induction of senescence and apoptosis when the damage cannot be repaired (Seviour and Lin 2010).

The members of the PI3K family ATM (ataxia telangiectasia mutated) and ATR (ATM-Rad3-related) play a distinct role in the DDR pathway. ATM is critical in the immediate response of cells to DSBs and the subsequent switch to ATR activation following DNA end resection. The checkpoint kinases Chk1 and Chk2 are widely considered to be the major effectors of the DDR in regulating cell cycle checkpoints and coordinating this with DNA repair. p53 can be phosphorylated by a number of DDR proteins, including ATM, ATR and Chk2. This phosphorylation reduces the binding of p53 to MDM2, resulting in stabilization and activation of the p53 protein. A key activity of p53 in the cell cycle is induction of expression of p21- regulator of both the G1/S and G2/M transitions acting as an inhibitor of cyclin-dependent kinases (CDK) (Figure 5).

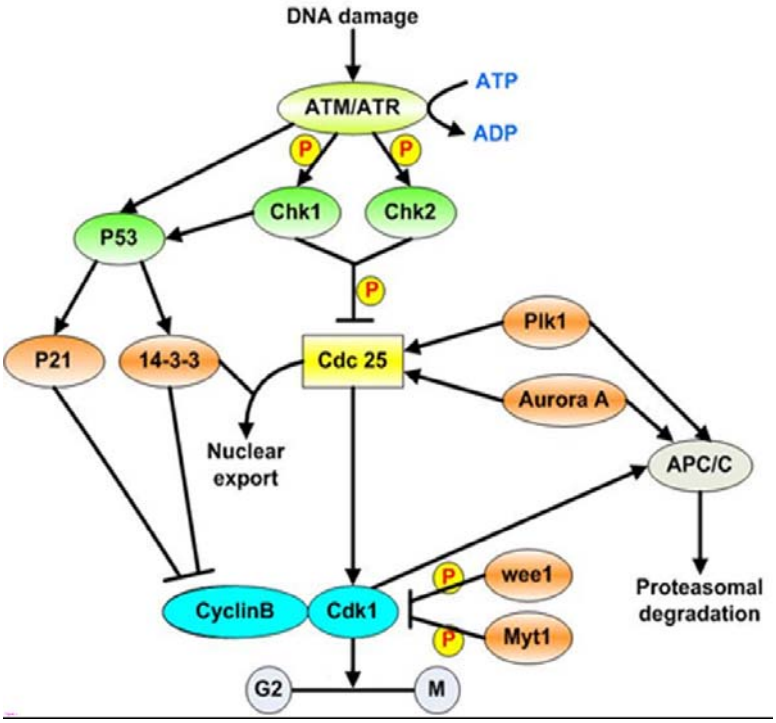

Figure 5. Activation of the G2/M checkpoint after DNA damage. In response to DNA damage, the ATM, ATR signaling pathway is activated, which leads to the phosphorylation and activation of Chk1 and Chk2

and to the subsequent phosphorylation of Cdc25. Phosphorylated Cdc25 is sequestered in the cytoplasm by 14-3-3 proteins, which prevents activation of cyclinB/Cdk1 by Cdc25 and results in G2 arrest. Activated ATM/ATR also activates p53-dependent signaling. This contributes to the maintenance of G2 arrest by upregulating 14-3-3, which sequesters Cdk1 in the cytoplasm. In addition, p53 induces the transactivation of p21, a Cdk inhibitor that binds to and inhibits cyclinB/Cdk1 complexes. P: phosphorylation. Adapted from Wang *et al.* 2009.

Centrosome is a cell structure responsible for integration of the DDR response. An increasing number of cancer related proteins have been shown to reside in or traffic in and out of centrosomes. These regulators include a number of cell cycle-regulated proteins, including cyclin B1, Cdks, Chks, Plks and Aurora kinases. In principle, checkpoint proteins at the centrosome could serve as points of cross-talk for DNA damage response and regulation of mitotic spindle. Alternatively, a centrosome is known to serve as a site of sequestration for inhibition of signaling or a meeting place for interacting proteins independent of spindle functions (Golan *et al.,* 2010).

G2M checkpoint is a major site of DNA repair in stem cells as the G1S checkpoint is absent. Moreover, under spindle damage ESC can easily overcome this checkpoint and enter reversible tetraploidy which is uncoupled from apoptosis (Mantel *et al.,* 2007).

In response to DNA damage, for double strand breaks repair cell can use mechanism of NHEJ (non-homologous end joining) in G1/S, but more certain after longer period of DNA damage is HR (homological recombination), that occurs in late S, G2 phase of the cell cycle (Takata 1998).

DNA damage caused by ionizing irradiation leads to phosphorylation of H2AX that is mediated by ATM, ATR and p53. γH2AX is involved in accumulation of DNA damage signaling and repair proteins at DSBs. The generation of DSBs triggers the relocalization of many DDR proteins such as MRE11/NBS1/RAD50, MDC1, 53BP1, and BRCA1 to nuclear foci where these proteins colocalize and interact with γH2AX. The γH2AX foci help keeping the broken DNA ends together and make repair more successful. Recruitment of cohesins to the site of DNA damage to promote sister chromatid-dependent recombinational repair DNA cohesion induced by double-strand DNA break and mediated by γH2AX has an important function during repair of double-strand breaks following DNA replication by holding the damaged chromatid close to its undamaged sister template (Podhorecka *et al.,* 2010).

1.7 Accelerated senescence induced by anti-cancer treatments

Cellular senescence was first described, when it was discovered that human cells derived from embryonic tissues in culture could only divide a finite number of times (40-60) (Hayflick and Moorhead, 1961). Replicative senescence is irreversible growth-arrested state of aged cells, triggered by telomere shortening and uncapping of telomere ends. Signals emitted by the telomere attrition are recognized by a cell similarly to the double-strand DNA break (DSB)-induced signals (d'Adda di Fagagna *et al.,* 2003). DNA damage response to telomere shortening-dependent senescence is mediated by the ATM/ATR-p53-p21 pathway (Herbig *et al.,* 2004).

Telomere length-independent senescence is termed accelerated senescence, premature senescence, stress- or aberrant signaling-induced senescence (STASIS), or extrinsic senescence (Itahana *et al.,* 2004, Dimri 2005). Accelerated senescence is induced by exposure to various DNA damaging and other cellular stresses (including inappropriate culture conditions). Activation of oncogenic and mitogenic signals, such as H-RAS, induces oncogene-induced senescence (OIS). Non-telomeric signals besides ATM/ATR-p53-p21 pathway, also induce the p16-pRB pathway of senescence in human cells (Dimri 2005).

Senescent cells can be identified by characteristic morphologic features including enlarged and flattened cell shape with increased cytoplasmic granularity, polyploidy, and

expression of the senescence marker, β-galactosidase (SA-β-gal) (Wu *et al.,* 2012) and accumulation of a distinct heterochromatin structures, termed senescence-associated heterochromatin foci (SAHFs) (Narita *et al.,* 2003). These cells are metabolically active, however cell division is arrested (Gey *et al.,* 2013).

Tumour cells are characterized by their ability to by-pass replicative senescence, however mounting evidence suggests that ACS is a tumor response to therapy (Wu *et al.,* 2012). For example, a study of breast carcinoma tumor patient samples after chemotherapy showed SA-b-gal expression in 41% of patients compared to only 10% of specimens from patients who underwent surgery alone (te Poele *et al.,* 2002). It is suggested, that senescence serves as natural barrier against tumorigenesis. However in fact, it is more likely that the barrier to cancer is cell cycle arrest, while senescence (in both cancer and normal cells) is rather a prerequisite for cancer (Blagosklonny 2011). Cancer and senescence have a lot in common. The pro-senescent phenotype due to overactivation of MAPK and PI3K/mTOR can be linked to hallmarks of cancer such as angiogenesis, apoptosis-avoidance, aerobic glycolysis, invasion and metastasis (Blagosklonny 2011).

Rare cancer cells can escape senescence after chemotherapy and restore proliferation which has been estimated to occur at a frequency of 1×10^6 cells. (Wu *et al.,* 2012). Although this event is relatively rare, it likely provides a notable number of resistant tumour cells capable to cause relapse of cancer desease in a patient.

1.8 Autophagy and its role in tumour growth and the phenomenon of the chromatin autophagy in the damage response of ETC

Autophagy is considered to be a survival mechanism with a protective function against stress conditions and in baseline level maintains homeostatic functions by removal and re-utilisation of peptidic aggregates, long-lived proteins and aging/damaged organelles in all normal healthy cells. Autophagy contributes to normal development, cellular differentiation and tissue remodeling. There are three forms of autophagy, that differ in function as well as in how cargo is delivered to the lysosome: chaperone-mediated autophagy (CMA), microautophagy and macroautophagy.

Macroautophagy (further - autophagy) begins with the formation of a double-membrane vesicle – autophagosome around the cargo macromolecules, that can consist of entire organelles, protein aggregates, nucleic acids, and lipids, as well as pathogens. Autophagosomes fuse with lysosomes to form autolysosomes where lysosomal enzymes degrade engulfed constituents. The end products of this degradation process are amino acids, fatty acids, and nucleotides, which are released back into the cytoplasm and recycled for energy metabolism. The stages of autophagy - initiation, elongation, closure, maturation, and degradation, are distinguished. These steps are regulated by the ATG (Autophagy-related - genes) proteins, such as ATG6 (Beclin-1), ATG8, ATG4, ATG12, ATG5, and ATG16. The important activator of autophagy is a decrease in nutrient availability which causes the inhibition of mTOR signalling (Rosenfeldt and Ryan 2011, Kimmelman 2013). Deregulated autophagy plays a major role in a wide array of human diseases including cancer. The binding of human oncogene BCL2 protein to the autophagy-related beclin product seems to inhibit the autophagy program (Liang *et al.,* 1999).

The autophagic pathway can be upregulated in response to a variety of stressful conditions and chemical triggers also including: glucose and/or amino acid deprivation; growth factor withdrawal; hypoxia; accumulation of unfolded proteins within the ER lumen; increased cytosolic Ca2+ concentrations; exposure to radio- and chemotherapy; inhibition of the cytosolic pool of the tumor suppressor protein p53. Autophagy may initially play a pro-survival role and later shift and interconnect with various forms of cell death (Morselli *et al.,* 2011), also mixed ones (Chaabane *et al.,* 2013).

Interestingly, there is evidence, that during tumour development, autophagy may be oncogenic in some contexts, but in others it facilitates tumour suppression (Figure 6). Autophagy serves as a barrier to tumor initiation, but once cancer is established, it appears that adaptive changes occur that now result in positive roles for autophagy in malignant progression and in subsequent tumor maintenance. Autophagy participates in tumour growth and maintenance by supplying metabolic substrate, limiting oxidative stress, and maintaining cancer stem cell population (Rosenfeldt and Ryan 2011, Kimmelman 2013, Lu and Harrison-Findik 2013). Rausch and colleages (2012) suggested that enhanced autophagy levels facilitate survival of CSC in hypoxic conditions.

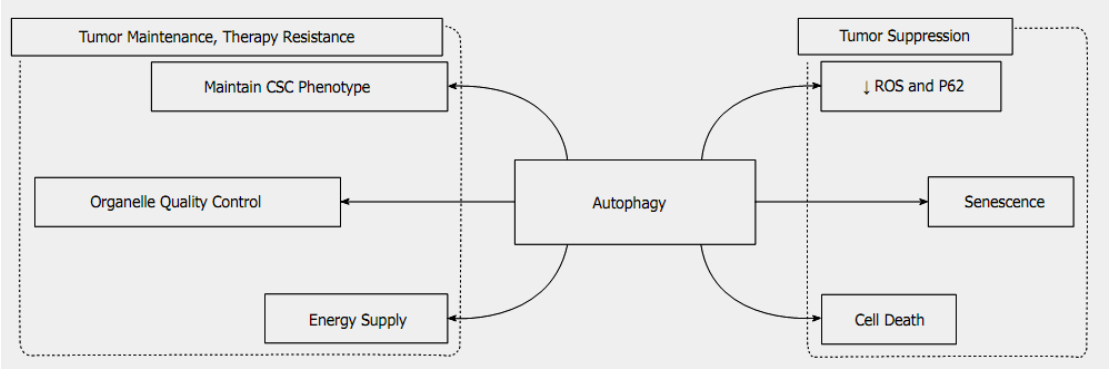

Figure 6. Schematic summary of the role of autophagy in cancer. Autophagy contributes to tumor suppression as well as tumor maintenance and therapy resistance. The mechanisms by which autophagy is involved in tumor suppression include limiting the accumulation of ROS and P62, and inducing senescence and cell death. On the other hand, autophagy facilities tumor maintenance and therapy resistance by providing the tumor with metabolic substrates and maintaining intracellular homeostasis (organelle quality control), and by possibly contributing to the maintenance of CSC phenotype. CSC: Cancer stem cell; ROS: Reactive oxygen species. Adapted fom Lu and Harrison-Findik 2013.

Regulation of DNA damage repair by autophagic process changing the acetylation status of key repair proteins was revealed (Robert *et al.*, 2011).

Autophagy is considered as a hallmark of cellular senescence (Vellai, 2009), it is activated upon acute induction of senescence (Young *et al.,* 2009). The activity of cellular senescence marker - SA-ß-gal and autophagy activation are well correlated during accelerated senescence, and it was found that, depletion of the autophagy-related genes ATG5 or ATG7 shows inhibition of SA-ß-gal activity during the oncogene (RAS) induced senescence.

In mammalian cells, macroautophagy is generally thought to affect the cytosol or cytoplasmic organelles, however degradation of the whole nuclei was described, for example, in filamentous fungi *Aspergillus oryzae* (Shoji *et al.,* 2010) and binuclear unicellular organism *Tetrahymena,* where the macronucleus is degraded by nucleolysis and autophagy and becomes extruded (Lu *et al.,* 2001). It was found in induced ETC, that a considerable portion of genetic material undergoes autophagic digestion and extrusion, indicating some form of sorting (Erenpreisa *et al.,* 2000). In recent research Rello-Varona *et al.* (2012) revealed removal of selected micronuclei by autophagic degradation and suggested a role of this process for the genome stabilizing effects.
The significance of these phenomena is unclear and needs further investigations.

2. Materials and Methods

2.1. Cell Culture and treatments

The Burkitt's lymphoma cell lines Namalwa and Ramos was obtained from the American Type Culture Collection (ATCC) and has an established TP53 mutant allele (O'Connor *et al.,* 1993). The human B-lymphoblastoid cell lines WI-L2-NS (TP53mutant -mt) and TK6 (TP53wild type-wt) were isolated from the same patient at different times during treatment and were obtained from Dr. P. Olive (Canada). Lymphoma cell-lines were maintained in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS; Sigma) at 37 °C in a 5 % CO2 humidified incubator.

HeLa S3 cells were grown in suspension under constant rotation in Joklik's MEM containing 10% heat-inactivated calf serum (Hyclone) and antibiotics. The adherent HeLa cells were cultured on glass cover slides in HAM-1 (Sigma) medium supplied with 10% FCS.

The Embryonal carcinoma cell line - human ovarian teratocarcinoma PA1 originated from the ATCC was obtained from the Vertebrate Cell Culture Collection of the Institute of Cytology (St-Petersburg, Russia), were cultured in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal calf serum. Cells were grown in 5% CO_2 in air incubators at 37°C.

For experimental studies, cells were maintained in log phase of growth for at least 24 hours prior to irradiation. Cells were irradiated with linear accelerator (Clinac 600 C, VarianMedical Systems) using a 4 MeV photon beam at a dose rate of 1 - 2 Gy/min.

Correspondingly, a single acute dose of 5 or 10 Gy was delivered in all experiments. In some experiments cells were treated with topoisomerase II inhibitor etoposide (ETO; Sigma) – 8 μM for 20 h or PXT (paclitaxel, Ebewe Pharma) – 50 nM for 20 h. After genotoxic treatment, cells were maintained by replenishing culture medium every 48-72 h. In certain experiments, retinoic acid (Sigma; 1 μM) was added to the culture medium for 48 h from day 3 to day 5 post-irradiation.

Smears of peripheral blood were used as a negative control for NANOG in immunofluorescence studies.

To determine the capacity of cells to replicate DNA, BrdU (5-bromo-2'-deoxyuridine) was added at 5 mM to the cell culture for 24 h or 10mM for 1h prior to fixation of cytospins with methanol.

2.2. Radiosensitivity test

Equal cell number was irradiated by a grade of irradiation dosages and seeded into 96 wells at different dilutions. The wells giving positive cell growth after 14 days were counted and the data presented as curves of surviving fraction at cell dilutions versus irradiation dosage (Gy).

2.3. RT-PCR

Total RNA was extracted from cells by using TRIZOL (Invitrogen) and treated with DNase I (Fermentas MBI). cDNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas MBI) according to the protocols of the manufacturer. The absence of contamination with chromosomal DNA was verified by PCR using primers DQA-1 and KIR3DL2. Expression of pluripotent cell markers was verified in comparison to embryonal stem cells cDNA (Millipore, SCR063). cDNA from peripheral blood lymphocytes (PBL) was kindly provided by Dr. HTC Chan, Southampton University, UK. Primers for PCR (Table 1) were designed using Primer 3 software; the primers for OCT4 B1 isoform were taken in

Atlasi *et al.* (2008). GenBank accession numbers of the templates used for design are as follows: OCT4-A (POU5F1), NM_002701; OCT4-B (POU5F1)_B, NM_203289; OCT4-B1 (POU5F1)_B1, EU518650; POU5F1B (POU5F1P1), NR_002304; OCT-4 ps4 (POU5F1P4), NC_000001; NANOG, NM_024865; SOX2, NM_003106. Amplification was carried out in a total volume of 50 µl with 1 – 4 µl cDNA in standard conditions using 0.5 units of Taq DNA polymerase (Fermentas MBI) with a BioCycler TC-S (BioSan). PCR conditions were as follows: 94 °C for 5 min (if not specified otherwise) 94 °C for 30 s, 56 – 60 °C for 20 s, 72 °C for 1 min; final extension step at 72 °C for 7 min. Amplified PCR products were resolved on 1.2 % agarose gels. The resulting PCR products fragments were analyzed by sequencing after ExoI/SAP treatment (Fermentas, MBI) using the fluorescent Big DyeTerminator v. 3.1 Cycle Sequencing protocol on a 3130xl Genetic Analyzer (Applied Biosystems). The structure of OCT4 splicing isoforms and location of the primer binding sites is presented in Figure 7.

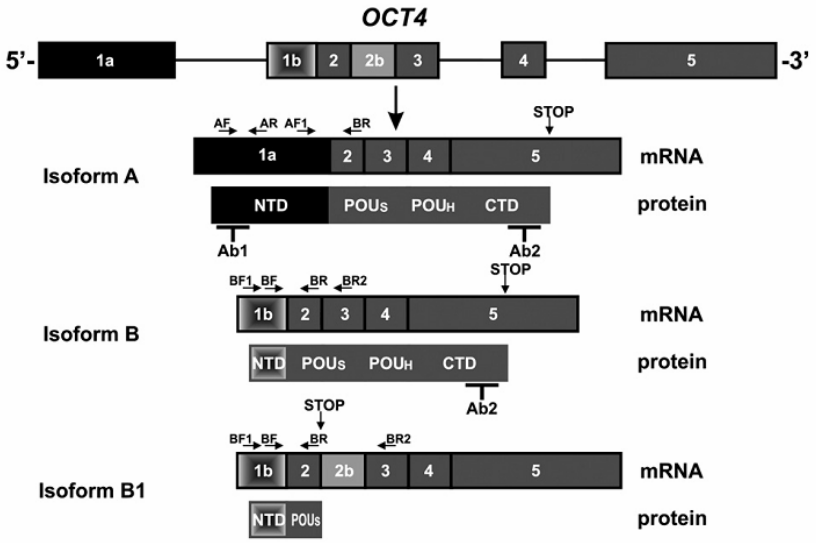

Figure 7. Structure of OCT4. Three different OCT4 isoforms are generated by alternative splicing which are schematically shown here with exons depicted as boxes. The RT-PCR primer binding sites and antibody binding epitope regions used in this study are indicated. POUs – POU-specific domain, POUH – POU homeodomain, NTD – N-transactivation domain, CTD – C-transactivation domain.

2.4. Western blotting

For Western blotting cells were lysed in lysis buffer (20 mM Hepes pH-7.9, 1 mM EDTA, 0.4 M NaCl, 1 mM DTT, Protease Inhibitor Cocktail (Sigma P8340), 1% NP-40). Shrimp alkaline phosphatase (AP; Fermentas) was added to certain protein extracts to assess the degree of phosphorylation (2 U per sample for 30 min at 37 °C).

Also for protein extraction, ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Lithuania), along with Protease Inhibitor Cocktail (Sigma P8340) was used, according to the manufacturers' instructions. The purity of isolated cell nuclei was assessed by immunofluorescence using the antibodies against Lamin B and α-tubulin. Equal protein loading in each lane was checked by Ponceau S staining. As loading and fraction purity controls antibodies against GAPDH were used (positive staining was observed in cytoplasmic but not nuclear lysates). Protein samples (10 µg) were separated by SDS PAGE on 9% gels and then blotted onto nitrocellulose membranes (Bio-Rad Labs). The membranes were blocked with 5% non-fat dry milk in PBS with 0.05% Tween 20 (Sigma) and primary antibodies applied overnight. The source of antibodies is presented in Table 2 and 3. To confirm specificity, a blocking peptide for the OCT4 rabbit polyclonal (ab20650) was mixed with primary antibody at 1 µg/ml and incubated at room temperature (RT) for 30 min before application. Detection was performed with HRP-conjugated secondary antibodies and ECL

(Amersham). The structure of OCT4 splicing isoforms and location of the epitopes for the two OCT4 antibodies used is presented in Figure 7.

2.5 Immunofluorescence (IF)

Cells were trypsinized, pelleted, washed in warm PBS, resuspended in FBS and cytospun on to polylysine-coated slides. For detailed cytological studies, the cells were also grown on glass coverslips. Standard IF staining was performed as follows: Fixation was performed in methanol −20 °C for 7 min (30min for γ-H2AX staining), followed by 10 short rinses in ice-cold acetone and semi-drying in air. Slides were then washed three times in TBS/0.01% tween-20 (TBS-T) (0.05% Tween-20 for γ-H2AX staining) before blocking in TBS/1% BSA/0.05% tween for 15 min. Primary antibody diluted in TBS/1% BSA/0.025% tween was added at over-night at 4 °C in a humidified chamber followed by three washes in TBS-T. Secondary antibody was added at room temperature for 40 min followed by three 5 min washes in TBS-T. Poststaining was with DAPI (0.25 μg/mL). Cells were finally embedded in Prolong Gold (Invitrogen). For MDC staining, cell cultures were incubated with 0.05 mM MDC at 37◦C for 1 h followed by fixation in 4% paraformaldehyde and two washes in PBS. The slides were counterstained with PI (propidium iodide; BD Biosciences Pharmingem), mounted into Permount (Thermo Fisher Scientific) and immediately imaged.

The sources and dilutions of the primary and secondary antibodies are listed in Table 2 and 3.

2.6 Microscopy

A fluorescence microscope (Leitz, Ergolux L03-10) equiped with a colour videocamera (Sony DX-S500) was used to examine cell preparations, record images and perform image cytometry. For three-colour images and colocalisation studies, the BRG filter system (Leica) providing nonoverlapping excitation and transmission emission of blue, red, and green bands was used. In addition, confocal microscopy (Leica, DM 600) was used with the images scanned in the three different colour channels in sequence.

2.7 DNA Image Cytometry

Cells grown on coverslips were rinsed in PBS and serum. Alternatively trypsinised cells were washed in warm PBS and suspended in FBS and cytospun onto glass slides. Both preparations were then fixed in ethanol/acetone (1:1, v/v) overnight at 4◦C and air dried. For stoichiometric DNA staining, slides were hydrolysed with 5N HCl for 20min and stained with 0.05% toluidine blue in McIlvain 50% buffer pH 4 for 10min at room temperature, rinsed, dehydrated in warm butanol, and passed through xylene prior to embedding in DPX (Sigma-Aldrich). DNA content was measured as the integral optical density in 200 cell nuclei in the green channel of the calibrated video camera, using Image Pro Plus 4.1 software (Media Cybernetics; REO 2001, Latvia). In parallel, optical density and nuclear area were registered. The estimated sum of device and measurement error was <5%. The 2C DNA content was determined by the modal value of the first G1 peak. Mitotic indices were counted per 1,000–2,000 cells in the same samples.

2.8. Two channel flow cytometry for OCT4 and DNA content

Cells were harvested at relevant time points, washed in cold TBS, and fixed overnight in cold (–20 °C) 70% ethanol. After two washes in TBS, cells were permeabilised with TBS/4% BSA/0.1% Triton X-100 for 10min at RT. After that, samples were incubated with rabbit polyclonal anti-OCT4 antibody solution (5 μg/ml) (ab19857) in TBS/4% BSA/0.1% Triton X100 for 1 h at RT. Following three washes in TBS, cells were incubated with goat

anti-rabbit Alexa Fluor 488 solution in TBS/4%BSA/0.1% Triton X100, 1:200 for 1 h in the dark. Then, DNA was counterstained with 10 μg/ml propidium iodide (PI) solution in PBS, containing 200 μg/ml RNAse (Sigma) and assessed by flow cytometry using a FACScan (BD Biosciences) using Cell Quest Pro Software.

2.9. Fluorescence in situ hybridisation (FISH)

HeLa cells grown on microscopic slides were treated for 20 min with 75mM KCl and fixed by five changes of methanol/glacial acetic acid (3:1). Commercial pericentric satellite DNA probes for chromosomes 10 and X were applied according to the manufacturer's instructions (Molecular Cytogenetics; Q-BIOgene). Cells were embedded under coverslips in Prolong Gold with DAPI (Invitrogen).

2.10. RNAi knock-down

REC8 expression was suppressed using shRNA plasmids (Origene; HuSH 29 mer). First, a panel of 4 plasmids were tested for their ability to down-regulate REC8-GFP in 293F cells following transient transfection (using 293 Fectin according to the manufacturer's instructions; Invitrogen) with REC8-GFP and shRNA. The two shRNA plasmids that most effectively reduced REC8-GFP expression (as judged by flow cytometry) were then co-transfected into Namalwa cells by electroporation (960 μF; 0.25 mV) and the cells were subjected to selection with puromycin 24 h later. After 12 days, surviving clones were expanded, sub-cloned and assessed for their level of REC8 expression by either immunofluorescence or Western blotting. Equivalent clones transfected with empty vector were produced as controls.

Table 1. Sequences of primers used for RT-PCR

Gene/primer	Forward primer sequence 5'→3	Reverse primer sequence 5'→3'
β-ACTIN	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG
DQA-1	GTGCTGCAGGTGTAAACTTGTACCAG	CACGGATCCGGTAGCAGCGGTAGAGTTG
KIR3DL2	CGGTCCCTTGATGCCTGT	GACCACACGCAGGGCAG
NANOG	CACCTACCTACCCCAGCCTT	CTCGCTGATTAGGCTCCAAC
OCT4-A – AF/AR	TTCTCGCCCCCTCCAGGT	TCAGAGCCTGGCCCAACC
OCT4-B/B1 – BF/BR	GAAGTTAGGTGGGCAGCTTG	AATAGAACCCCCAGGGTGAG
OCT4-B/B1 – BF1/BR2	AGACTATTCCTTGGGGCCACAC	CTCAAAGCGGCAGATGGTCG
POU5F1B (OCT4-ps1)	AGGCCGATGTGGGGCTCAT	CCAGAGTGATGACGGAGACT
OCT4-ps4 – AF1/BR	AGGTTGGAGTGGGGCTAGTG	AATAGAACCCCCAGGGTGAG
SOX2	ACCTACATGAACGGCTCGC	CCGGGGAGATACATGCTGA
(SGOL1/ SGO1	AACCTGCTCAGAACCAGG	TCCTGGAAGTTCAGTTTC
SGOL2/SGO2	CAGTCTTTCTGAGTTCCA	GTTTCATTAGTATGACCAT

Table 2. Primary antibodies

TARGET	Description	Specifity/Immunogen	Product nr and Manufacturer	Use
OCT4A	Mouse monoclonal	Peptide raised against amino acids 1-134 of OCT-3/4 of human origin non	sc-5279, Santa Cruz	W, IF

		cross-reactive with OCT-3/4 isoforms B and B1.		
OCT4A/B	Rabbit polyclonal, ChIP Grade	Peptide derived from within from within residues 300 to the C-terminus of human OCT4.	ab19857, Abcam	W, IF, F
OCT4B	Goat polyclonal	Raised against a peptide mapping near the N-terminus of Oct-3/4 of human origin	sc-8630, Santa Cruz	IF
NANOG	Rabbit polyclonal	Epitope corresponding to amino acids 151-305 mapping at the C-terminus of Nanog of human origin.	sc-33759, Santa Cruz	W, IF
NANOG	Mouse monoclonal, clone NNG-811	Against human NANOG.	N3038, Sigma	IF
P16INK4A	Rabbit polyclonal	Raised against a peptide mapping at the N-terminus of P16 of human origin.	sc-467, Santa Cruz	IF
Aurora B kinase	Rabbit polyclonal	Peptide derived from within residues 1 - 100 of Human AURORA B.	ab2254, Abcam	IF
α-Tubulin	mouse monoclonal	Recognizes an epitope located at the C-terminal end of the α-tubulin isoform in a variety of organisms.	T5168, Sigma	IF
γ-H2AX	Rabbit polyclonal	Recognizes mammalian, yeast, D. melanogaster, and X. laevis γ-H2AX.	4411-PC-100, Trevigen	IF
RAD51	Mouse monoclonal	Targeted to amino acids 1-138 of human RAD51.	ab213, Abcam	IF
GAPDH	Mouse monoclonal, clone 6C5	Rabbit muscle GAPDH.	ab8245, Abcam	W
REC8	Goat polyclonal	Raised against a peptide mapping near the N-terminus of REC8 of human origin	sc-15152, Santa Cruz	IF, W
REC8	Rabbit polyclonal	Raised against residues near the C terminus of human REC8	ProteinTech Group) 10793-1-AP	IF, W
NuMa	Mouse monoclonal	Nuclear matrix from human ME-180 cervical carcinoma cells, epitope within amino acids 658-691	107-7, Calbiochem	IF
centromere/ kinetochore	Human polyclonal	Derived from human CREST patient serum.	15-234, Antibodies Inc	IF
DMC1	Mouse monoclonal	Specific for DMC1 - does not cross-react with the related protein Rad51.	ab-11054, Abcam	IF
RAD52	Goat polyclonal	epitope mapping at the C-terminus of RAD52 of human origin.	sc-7674, Santa Cruz	IF
BrdU	Mouse monoclonal	Reacts with Bromodeoxyuridine.	MS-1058-P; Lab Vision	IF
β-TUBULIN	Mouse monoclonal, clone DM1B	Native chick brain microtubules.	Neomarkers	IF
CATHEPSIN B	Rabbit polyclonal	Full length native protein, recognises liver Cathepsin B. Does not crossreact with Cathepsin L or Cathepsin H.	ab30443, Abcam	IF
PML	Mouse monoclonal	N-terminal epitope corresponding to residues 37-51 of PML of human origin.	sc-966, Santa Cruz	IF
SOX2	Rabbit polyclonal	Epitope corresponding to amino acids 131-195 of SOX2 of human origin.	sc-20088x, Santa Cruz	IF
LaminB	Goat polyclonal	Epitope mapping at the C-terminus of Lamin B1 of human origin.	sc-6216, Santa Cruz	IF
LC3B	Rabbit polyclonal	Peptide derived from within residues 1 - 100 of human LC3B.	ab63817, Abcam	IF

Table 3. Secondary Antibodies

Antibody	Conjugate	Product nr and Manufacturer	Use *
Goat anti-mouse IgG	Alexa Fluor 488	A31619, Invitrogen	IF
Goat anti-mouse IgG	Alexa Fluor 594	A31623, Invitrogen	IF
Goat anti-rabbit IgG	Alexa Fluor 488	A31627, Invitrogen	IF
Goat anti-rabbit IgG	Alexa Fluor 594	A31631, Invitrogen	IF
Rabbit anti-goat IgG	Cy3	C2821, Sigma	IF
Donkey anti-goat IgG	Alexa Fluor 594	A-11058, Invitrogen	IF
Chicken anti-rabbit IgG	Alexa Fluor 488	A-21441, Invitrogen	IF
Donkey anti goat IgG	Alexa Fluor 488	A11055, Invitrogen	IF
Chicken anti-rabbit IgG	Alexa Fluor 594	A-21442, Invitrogen	IF
Chicken anti-mouse IgG	FITC	sc-2989, Santa Cruz	IF
sheep anti-human IgG	FITC	produced by Mark Cragg	IF
Rabbit anti-mouse IgG	HRP	61-6520, Invitrogen	W
Goat anti-rabbit IgG	HRP	32460,Thermo Fisher Scientific	W

*W=Western, IF=Immunofluorescent staining, F=flow cytometry.

3. Results

The results are presented here as original publications and as unpublished results.
The author's contribution to the publications:

Original paper I
Erenpreisa J, Cragg MS, Salmina K, Hausmann M, Scherthan H. The role of meiotic cohesin REC8 in chromosome segregation in gamma irradiation-induced endopolyploid tumour cells. Exp Cell Res. 2009, 315:2593-603
Contribution: participated in experimental design, performed immunocytochemistry, Western blott analysis, microscopy and analysis of results, participated in the manuscript draft preparation.

Original paper II

Salmina K, Jankevics E, Huna A, Perminov D, Radovica I, Klymenko T, Ivanov A, Jascenko E, Scherthan H, Cragg M, Erenpreisa J. Up-regulation of the embryonic self-renewal network through reversible polyploidy in irradiated p53-mutant tumour cells. Exp Cell Res. 2010;316:2099-112. Epub 2010 May 10.
Contribution: did most of the experimental design, carried out most of immunocytochemistry, Western blotting, microscopy and analysis of results, prepared the draft manuscript and its final version.

Original paper III

Erenpreisa Je, Salmina K, Huna A., Kosmacek EA, Cragg MS, Ianzini F, Anisimov AP. Polyploid tumour cells elicit para-diploid progeny through de-polyploidising divisions and regulated autophagic degradation. Cell Biol Int. 2011; 35:687-95**.**
Contribution: participated in experimental design, carried out most immunocytochemistry and analysis of results, participated in the manuscript draft preparation and its edit.

Review article IV

Erenpreisa J, Huna A, Salmina K, Jackson T and Cragg MS. Macroautophagy-aided elimination of chromatin: Sorting of waste, sorting of fate? Autophagy 2012; 8:1-5.
Contribution: participated in the manuscript draft preparation, design of the figures and search of the literature references.

3.1. Characterisation of the radiosensitivity of the studied lymphoma cell lines

The main experiments were carried out on HeLa- cervical carcinoma and three human lymphoma cell lines, Namalwa, WI-L2-NS and TK6. The radiosensitivity test performed in tripple on lymphomas showed that p53 mutant cell lines Namalwa and WI-L2-NS are radioresistant, while the TP53 wild-type TK6 (a parental cell line of WI-L2-NS) is radiosensitive (Figure 8A). WI-L2-NS (a TP53 mutant counterpart of TK6 derived from the same patient) displays 10-fold higher polyploidy and two-fold less apoptosis than the wt TP53 TK6 cells in the response to irradiation.

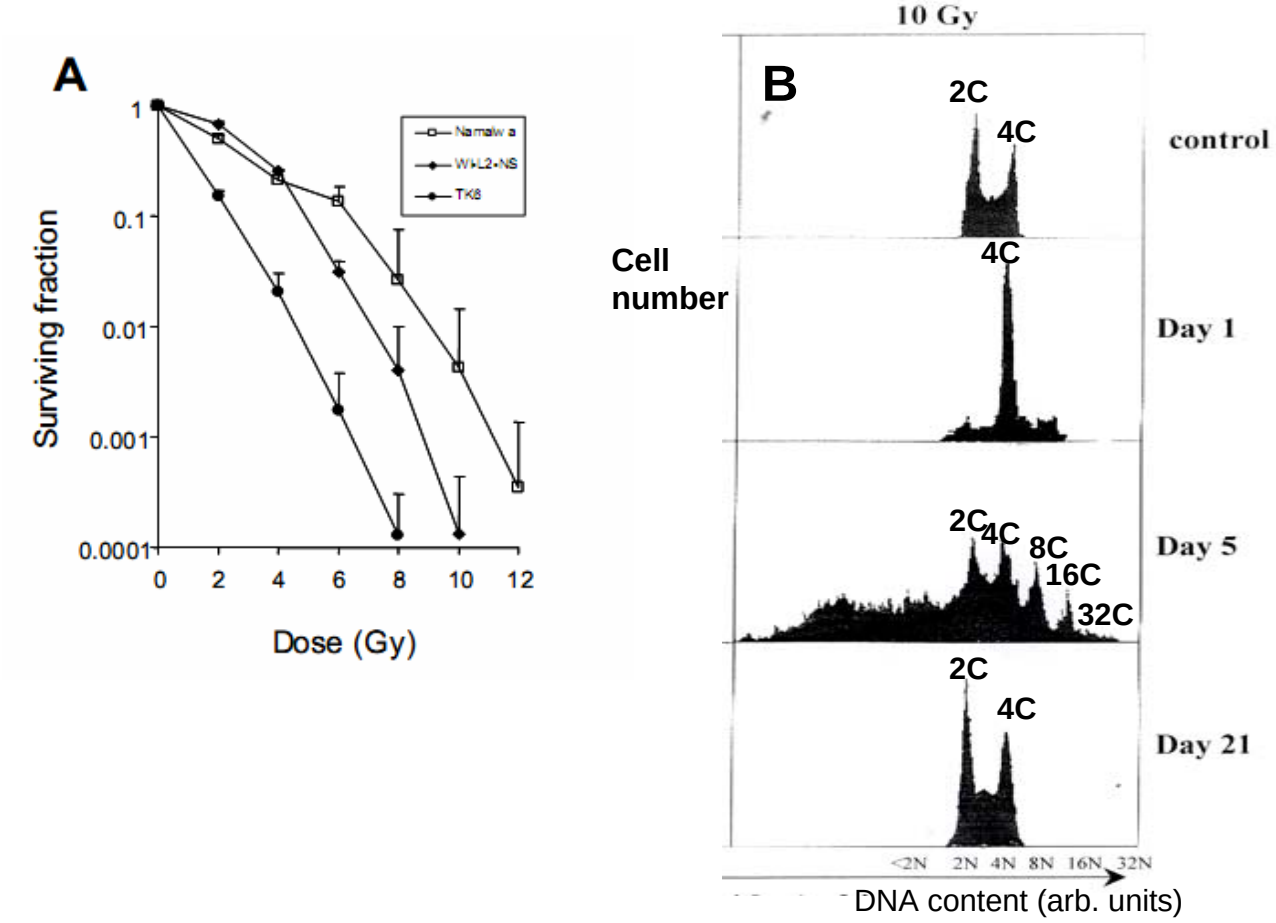

Figure 8. (A) Radiosensitivity assay of human lymphoma cell lines Namalwa, WI-L2-NS, TK6. (B) Typical dynamics of tumour cell ploidy after 10 Gy irradiation in the time-course showing G2 arrest followed by transient polyploidy as seen from propidium iodide DNA flow cytometry. Burkitt's lymphoma cell line Namalwa.

3.2. Typical dynamics of response of the TP53 mutated cells to irradiation by transient polyploidy.

As found previously (Illidge *et al.,* 2000; Erenpreisa et al., 2000; Puig *et al.*, 2008), the generation of transient endopolyploid tumour cells (ETCs) after genotoxic damage represents a potential survival strategy of tumour cells. Typical changes of cell cycle after irradiation are seen on Figure 8B. After irradiation induced G2-arrest for one-two days, the cells enter aberrant, often bridged mitoses which either undergo death by mitotic catastrophe or, become tetraploid through 'mitotic slippage' or bi-nucleation. Large amount of cells also undergo cell death trough apoptosis (seen as a sub-G1 fraction Figure 8B). Polyploidisation continues 3–5

days, leading to a ploidy-increase of up to 8–32C. On days 5–6 post irradiation bi-polar and multi-polar cell divisions start depolyploidisation. From days 7–9 onwards death of most giant cells can be observed but a small proportion of giant survivors give rise to small colonies of viable para-diploid tumour cells proven by isolation of the polyploidy fraction (Illidge *et al.,* 2000).

3.3 The role of the ectopically expressed meiotic cohesin REC8 in chromosome segregation in gamma irradiation-induced endopolyploid tumour cells.

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Research Article

The role of meiotic cohesin REC8 in chromosome segregation in γ irradiation-induced endopolyploid tumour cells

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ABSTRACT

Escape from mitotic catastrophe and generation of endopolyploid tumour cells (ETCs) represents a potential survival strategy of tumour cells in response to genotoxic treatments. ETCs that resume the mitotic cell cycle have reduced ploidy and are often resistant to these treatments. In search for a mechanism for genome reduction, we previously observed that ETCs express meiotic proteins among which REC8 (a meiotic cohesin component) is of particular interest, since it favours reductional cell division in meiosis. In the present investigation, we induced endopolyploidy in *p53*-dysfunctional human tumour cell lines (Namalwa, WI-L2-NS, HeLa) by gamma irradiation, and analysed the subcellular localisation of REC8 in the resulting ETCs. We observed by RT-PCR and Western blot that REC8 is constitutively expressed in these tumour cells, along with SGOL1 and SGOL2, and that REC8 becomes modified after irradiation. REC8 localised to paired sister centromeres in ETCs, the former co-segregating to opposite poles. Furthermore, REC8 localised to the centrosome of interphase ETCs and to the astral poles in anaphase cells where it colocalised with the microtubule-associated protein NuMA. Altogether, our observations indicate that radiation-induced ETCs express features of meiotic cell divisions and that these may facilitate chromosome segregation and genome reduction.

Introduction

When challenged with high doses of ionising radiation tumour cells can escape cell death by transient endopolyploidisation [1]. While most of these polyploid cells will undergo cell death following aberrant mitosis (mitotic catastrophe) [2] some will undergo genome reduction giving rise to viable para-diploid tumour cells [1–4]. Evidence from two different laboratories using various human tumour cell lines and DNA damaging agents (gamma irradiation and cisplatin) showed that FACS-isolated endopolyploid cells are capable of undergoing de-polyploidisation and producing a limited number of para-diploid clones [1,5]. These observations have been confirmed using direct time-lapse imaging [5–11]. Furthermore, the resulting mitotic cells often display elevated resistance to genotoxic treatment and are genomically altered [5,10].

Somatic reduction divisions (de-polyploidisation), were first described in *Culex* [12], and although not a frequent phenomenon, were found in a wide variety of taxons (reviewed in [13,14]).

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Abbreviations: MT, microtubule; ETCs, endopolyploid tumour cells; DSB, double-strand break

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Table 1 – Essential cell cycle and genetic events associated with mitotic catastrophe leading to birth and genome reduction in endopolyploid tumour cells.

Days after irradiation	Cell cycle events	Transcription/translation of meiotic and related genes
1	G2 arrest	Tyr P-15 CDK1 ↓[a] γH2AX ↑[a]
2–3	Aberrant mitoses	Tyr P-15 CDK1 ↑[a], MOS ↑↑[b], MAPK ↑↑[b]
3–4–5	Endopolyploidisation	MOS ↑[b,c], REC8 ↑[b,e,f], γH2AX ↑, DMC1 ↑[b,e,f], RAD51 ↑[a,f], AUR B ↑[d]
5–6	De-polyploidisation (reduction division?)	MOS ↓[b,c], REC8 ↑↑[b,e,f], STAG3 ↑[b], γH2AX ↑[a,f], DMC1 ↑[b,e,f], RAD51 ↑[a,f], AURKB ↑↑[d]
7–9	Senescence of polyploid cells and clonogenic growth of para-diploid cells	REC8 ↓[b,e], STAG3 ↓[b], γH2AX ↓[a,e], DMC1 ↓[b,e,f], RAD51 ↓[a,f], AURKB ~[d]

'~': remains positive in some giant cells.
[a] [43].
[b] [26].
[c] [42].
[d] [41,43].
[e] [8].
[f] Present study.

Nevertheless, their mechanism is still far from being well understood [15,16] and it has been hypothesized that somatic reduction may involve evolutionary features of meiosis (reviewed in [17]). The mechanism of somatic reduction in human tumour cells is currently under discussion [4,18,19] and its potential link to developmental processes has recently been proposed [20,21].

Polyploidy with associated genome instability is known as a source of genetic variation, which is thought to have served as an engine of macroevolution [16,22]. Spontaneous polyploidy and its subsequent reduction are thought to be involved in evolution of various forms of meiosis starting from asexual reproduction and parasexual cycles like cycling polyploidy [23–25].

Therefore, it is of interest that we found that radiation-induced ETCs of human lymphoma cell lines ectopically express meiotic proteins [17,26] such as the meiotic cohesin REC8, a feature that also occurs in human cancer cell lines originating from cervix (HeLa), breast (MDA-MB435) and colon (HCT116) [8]. Further upregulation of REC8 transcription follows irradiation in tumour cell lines of various origins [8].

REC8 belongs to the cohesin protein complex, which is essential for correct chromosome disjunction and homologous recombination in the mitotic and meiotic cycle [27]. In meiotic cells of budding yeast the REC8 cohesin component replaces mitotic cohesion SCC1 in the four cohesin complex and contributes to meiotic chromosome structure and homologue segregation [28–31].

REC8, together with Shugoshin 1 (SGOL1/SGO1), facilitates the reductional cell division in meiosis (reviewed in [32]). The central peculiarity of the first meiotic cell division is the segregation of homologous chromosomes, instead of sister chromatids, to opposite spindle poles thereby reducing the chromosome number. For this division to occur, there are two prerequisites: homologous chromosomes (further termed 'homologs') must first be paired and cross-linked by recombination and secondly, sister chromatids of each homolog must be cohesed to allow for homolog disjoining during the first meiotic division, with both of these processes requiring REC8 [33]. During the two meiotic divisions, REC8 is released from chromosome arms and centromeres consecutively [34]. The stepwise release of cohesion depends on SGO1 which stabilises REC8-mediated cohesion at sister centromeres in the first meiotic division [35–37]. The meiotic pairing of homologs is dependent on the formation of physiological DSBs by the SPO11 transesterase and their subsequent repair by homologous recombination [38] which involves, among others, the meiosis-specific recombinase DMC1 [39,40]. The central feature of the chromosome reduction in meiosis is omission of the S-phase between two meiotic divisions, thus providing for reduction of the chromosome number.

Previously, we have demonstrated that acute exposure of ETCs to ionising radiation induces exit from the mitotic cycle, altered DNA repair and gene expression changes that involve the upregulation of meiosis-specific genes such as REC8, MOS and SPO11 [8, 26]. A single 10 Gy dose of photon irradiation (IR) to p53 function-deficient cell lines causes them to undergo extensive changes in cell cycle progression so that they transiently form endopolyploid giant cells (Table 1) [1,41–43]. After G2-arrest for one to two days, the cells enter aberrant, often bridged mitoses and then either undergo mitotic death or, through 'mitotic slippage' or bi-nucleation, become tetraploid. Cycles of polyploidisation continue in the large proportion of cells on days 3–5, eventually leading to a ploidy-increase of up to 8–32C (polyploidisation phase). On days 5–6 post irradiation, this phase is switched to de-polyploidisation and bi-polar and multi-polar cell divisions occur. From days 7–9 onwards death of most giant cells can be observed and cell divisions of ETCs give raise to small colonies of para-diploid cells (Table 1; [8]).

From these changes it can be deduced that the most critical events determining the switch from polyploidisation to ploidy reduction in giant ETCs occur during days 5–6 post IR, a time frame when the viable cell population consists nearly exclusively of ETCs [1]. At this critical point MOS (which may mediate metaphase arrest in polyploid mitotic cells [44]) is becoming downregulated and the meiotic cohesin REC8 is expressed at increasing rate [8,17,26,42] (summarised in Table 1). During this time there also is a notable upregulation in the expression of Aurora B-kinase (AURKB), a core component of the chromosomal passenger complex, whose enhanced activity might play a role in ETC cell divisions [41] (Table 1). AURKB has recently been shown to play a role in sister chromatid cohesion and homolog segregation in meiosis [45–47]. Sister chromatid cohesion in turn depends on the REC8 cohesin (reviewed by [48]).

For the reasons above, we here investigated the expression and sub-cellular localisation of REC8, the mode of DNA synthesis and of chromosome segregation in irradiation-induced ETCs.

Materials and methods

Cell lines and irradiation of cells

The Burkitt's lymphoma cell line Namalwa was obtained from the American Type Culture Collection (ATCC) and has an established p53 mutant allele [49] (own unpublished observations). The WI-L2-NS human lymphoblastoid cell line was obtained from Dr. P. Olive (Canada) and is also p53 mutated [50]. These cell lines were maintained in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS; JRH Biosciences) at 37 °C in a 5% CO_2 humidified incubator. HeLa cells were obtained from Dr. F. Ianzini and were grown on slides in HAM-1 medium supplied by 10% FCS (Sigma) and penicillin/streptomycin at 37 °C in a 5% CO_2 humidified incubator. For experimental studies cells were maintained in log phase of growth for at least 24 h prior to irradiation. Suspension cells were irradiated at a density of 5×10^5 cells/ml; adherent cultures at ~60% sub-confluence. Cell lines were irradiated either with a Gulmay D3 225 X-ray source at a dose rate of 0.77 Gy/min or with a gamma-ray source — Linear accelerator, Clinac 600 C, Varian Medical Systems, USA 4 MeV photon beam at a dose rate of 1 Gy/min. After irradiation, cells were maintained by replenishing culture medium each 48–72 h. For DNA replication studies, bromodeoxyuridine (BrdU, 5 μM final conc.; Sigma) was added to the cultures of Wi-L2-NS or Namalwa cells at for 1, 10–13 and 19–20 h before harvest. In some experiments, Nocodazole (Sigma) was added (0.75 μM final conc.) 2 h prior to harvest. Finally, the cells were treated with 75 mM of KCl before fixation in methanol or methanol/acetic acid (3:1). Slides were prepared with a cytocentrifuge or spread on glass slides. For studies of the protein turn-over the following protease inhibitors were used: 25 μM calpain inhibitor, 5 μM Mg-132, and 10 μM lactocystin, added to the culture medium for 2 h or 24 h before cell harvest.

RT-PCR

RT-PCR was performed as described [26]. Shugoshin 1 (*SGOL1*/*SGO1*) was detected by the following primer pair: fwd: AACCTGCT-CAGAACCAGG; rev: TCCTGGAAGTTCAGTTTC, which reveals all six splicing forms. Shugoshin 2 (*SGOL2*/*SGO2*) was amplified by the primer pair: fwd: CAGTCTTTCTGAGTTCCA; rev: GTTTCATTAGTATGACCAT.

RNAi knock-down of REC8

REC8 expression was suppressed using shRNA plasmids (Origene; HuSH 29 mer). First, a panel of 4 plasmids were tested for their ability to down-regulate *REC8*-GFP in 293F cells following transient transfection (using 293 Fectin according to the manufacturer's instructions; Invitrogen) with *REC8*-GFP and shRNA. The two shRNA plasmids that most effectively reduced REC8-GFP expression (as judged by flow cytometry) were then co-transfected into Namalwa cells by electroporation (960 μF; 0.25 mV) and the cells were subjected to selection with puromycin 24 h later. After 12 days, surviving clones were expanded, sub-cloned and assessed for their level of REC8 expression by either immunofluorescence or Western blotting. Equivalent clones transfected with empty vector were produced as controls.

Western blotting

For Western blotting cells were lysed in lysis buffer (20 mM Hepes pH-7.9, 1 mM EDTA, 0.4 M NaCl, 1 mM DTT, Protease Inhibitor Cocktail (Sigma P8340), 1% NP-40). Shrimp alkaline phosphatase (AP; Fermentas) was added to certain protein extracts to assess the degree of phosphorylation (2 U per sample for 30 min at 37 °C). Protein samples were separated by SDS-PAGE and then blotted onto PVDF membranes (Hybond P, Amersham Biosciences or Bio-Rad Labs). The membranes were blocked with 5% non-fat dry milk

Table 2 – Antibodies: source and usage.

Primary antibodies	Secondary antibodies (dilution, if not stated otherwise, 1:400)
Goat polyclonal anti-hREC8 (E-18: sc-15152) 1:50 Blocking protein (sc:151152p) (×10)	Rabbit a-goat-IgG-Cy3 (Sigma) 1:400 Donkey a-goat IgG-Alexa Fluor 594 (Invitrogen)
Rabbit polyclonal anti-hREC8 (ProteinTech Group) 10793-1-AP) 1:50	Goat a-rabbit-IgG-Alexa Fluor 594; chicken a-rabbit-IgG-Alexa Fluor 488 and 594 (Invitrogen)
Monoclonal mouse anti-a-tubulin (Sigma, B-512) 1:2000	Goat a-mouse IgG-Alexa Fluor 488 and 594 (Invitrogen); chicken anti-mouse-IgG-FITC (sc-2989)
Monoclonal anti-hNuMa (Ab-2) Mouse mAb (107-7) (Calbiochem) (1:100)	Goat a-mouse IgG-.Alexa Fluor 480 or 594 (Invitrogen); chicken anti-mouse-IgG-FITC (sc-2989)
Human anti-h-centromere/kinetochore (Antibodies Inc; 15-234), (1:100)	sheep a-h-IgG-FITC (Dr. MS Cragg) (1:50)
Monoclonal mouse anti-hDMC1 (ab-11054) 1:100; does not cross-react with Rad51 http://www.abcam.com/index.html?datasheet=11054	Goat a-mouse IgG-.Alexa Fluor 480 or 594, Invitrogen; chicken anti-mouse-IgG-FITC (sc-2989)
Goat polyclonal anti-hRad52 (C-17):sc-7674 (1:50)	Rabbit a-goat- IgG -Cy3 (Sigma) 1:500; donkey a-goat IgG-Alexa Fluor 594 (Invitrogen)
Monoclonal mouse anti-hRad51 (NeoMarkers; 51RAD01; LabVision (UK) Ltd.) (1:100)	Goat a-mouse IgG-Alexa Fluor 480 or 594, Invitrogen; chicken anti-mouse-IgG-FITC (sc-2989)
Rabbit polyclonal anti-h-γH2AX (Trevigen; 4411-PC-020) (1:150)	goat a-rabbit- IgG-Alexa Fluor 480 or 594; chicken a-rabbit-IgG-Alexa Fluor 488 and 594 (Invitrogen)
Monoclonal mouse anti-BrdU (MS-1058-P; Lab Vision (UK) Ltd.) (1:100)	Goat a-mouse IgG-Alexa Fluor 488 and 594 (Invitrogen)

in PBS with 0.05–0.1% Tween 20 (Sigma) and then probed with the polyclonal antibody against REC8 (ProteinTechgroup Cat Nr 10793-1-AP) or β-actin (Sigma or Abcam) as a loading control. Detection was performed with HRP-conjugated secondary antibodies (anti rabbit-HRP) and ECL (both from Amersham Biosciences or Pierce).

Cytological staining

Cytospins or metaphase spreads were fixed in ethanol/acetone (1:1) and, after hydrolysis with 5 N HCl at 21 °C for 20 min and thorough rinsing in distilled water, were stained for DNA with 0.05% Toluidine blue in 50% McIlvain buffer, pH-4 for 10 min.

Fluorescence in situ hybridisation (FISH)

HeLa cells grown on microscopic slides were treated for 20 min with 75 mM KCl and fixed by five changes of methanol/glacial acetic acid (3:1). Commercial pericentric satellite DNA probes for chromosomes 10 and X were applied according to the manufacturer's instructions (Molecular Cytogenetics; Q-BIOgene). Cells were embedded under coverslips in Prolong Gold with DAPI (Invitrogen).

Immunofluorescence (IF)

Standard IF staining was performed as follows: Fixation was performed in methanol −20 °C for 10 min, followed by 10 washes in ice-cold acetone and semi-drying in air. Slides were then washed once in TBS and three times in TBS/0.01% tween-20 (TBS-T) before blocking in TBS/1% BSA/0.05% tween (TBT) for 15 min. NB: for REC8 immunostaining 5% serum was also added. Primary antibody was added at 37 °C for 1 h or over-night at 4 °C in a humidified chamber followed by three washes in TBS-T. Secondary antibody was added at room temperature for 40 min followed by two 5 min washes in TBS-T, one wash in TBS and embedding in Prolong Gold with DAPI (Invitrogen). The protocol for determination of incorporated BrdU by anti-BrdU-specific antibody included preceding denaturation of DNA by 2 N HCl at room temperature for 20 min. The primary and secondary antibodies used are indicated in Table 2.

Microscopy

A confocal laser microscope (Leica, DM 600) or fluorescence light microscope (Leitz, Ergolux L03-10) was used to examine the preparations and record images.

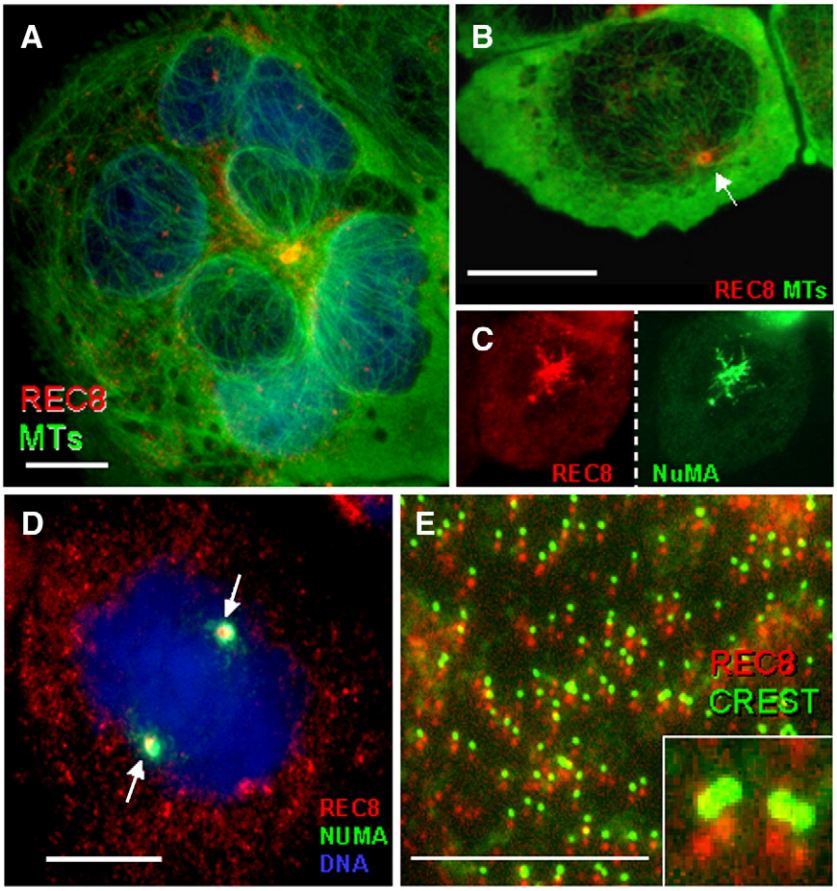

Fig. 1 – Association of REC8 with centrosomes in endopolyploid Namalwa cells after γ irradiation: (A, B) on days 3–4 post IR REC8 (red) forms a distinct signal at the interphase centrosome marked by converging microtubules (MTs, α-tubulin, green); (C) two colour channel image of an ETC stained for REC8 (red) and NuMA (green) displaying their colocalisation. (D) REC8 (red) forms a distinct signal spot (arrowed) at each spindle pole of a metaphase cell, which is surrounded by NuMA-positive material (green) in a cell at day 5 post IR. DNA is shown in blue (DAPI). (E) Chromatin of giant cell day 5 post IR displaying REC8 (red) protein spots colocalising with kinetochores (CREST, green). Boxed and enlarged insert is a typical pattern of kinetochore doublets and REC8 signals. The red and green foci are offset to better demonstrate their colocalisation. Bars = 10 μm.

Results

REC8 localises to sister centromeres, interphase centrosomes and spindle poles in dividing ETC

To investigate the potential role of REC8 in genome de-polyploidisation in ETCs, we investigated its expression and localisation by IF. Non-treated cells displayed a diffuse REC8 staining pattern in nuclei and cytoplasm (not shown). On days 3–4 post IR large mononuclear and multinuclear ETCs appeared and exhibited granular REC8 staining throughout the cells with accumulation at the centrosome (Figs. 1A, B). Four days after IR, lymphoid cell line ETCs displayed REC8 signals that colocalised with CREST anti-kinetochore serum (Fig. 1E). This pattern is reminiscent of the centromeric localisation of REC8 seen during anaphase I – metaphase II of meiosis [28]. On days 5–6 post IR, ETCs began to escape metaphase arrest and entered a phase of cell divisions as reflected by the appearance of bi-polar and multi-polar metaphases and anaphases. In metaphase ETCs, we found each centromere signal to colocalise with a REC8-positive spot as observed by confocal microscopy (Fig. 2A, insert) unlike in interphase ETCs [26], where co-staining of REC8 and kinetochore proteins is less frequent or irregular. In polyploid metaphase cells, REC8 also appeared at the spindle poles (Figs. 1C, D). Furthermore, in many early ETC metaphases REC8 formed a ring-like structure in addition to its centromeric localisation (Fig. 2A). Such structures have previously been observed with NuMA, a nuclear microtubule-binding protein that binds to the separating centrosomes during early metaphase and until the onset of anaphase where it contributes to the organization and stabilisation of microtubules in astral spindle poles [51]. Co-staining disclosed the almost perfect colocalisation of both REC8 and NuMa to these metaphase spindle poles (Figs. 1C, D).

During anaphase, ETCs also displayed REC8 on the spindle poles that occasionally displayed split signals, while centromere localisation was reduced or absent (Fig. 3). However, when present at centromeres REC8 staining was located between kinetochores signals (Fig. 3A, insert), a location that would be compatible with a role in mediating sister centromere cohesion.

To validate our IF observations, we performed control experiments in the presence of a 10-fold excess of REC8 blocking peptide in the anti-REC8 antibody solution: Cells stained with this mixture failed to display the discrete REC8 IF signals at kinetochores and spindle poles, while the cytoplasmic staining was reduced (suppl. Fig. S1), demonstrating the specificity of the antibody used. Furthermore, the REC8 expression and localisation to centromeres and centrosomes in ETCs was similar when two different polyclonal antibodies to REC8 were used along with appropriate secondary antibodies (Table 2).

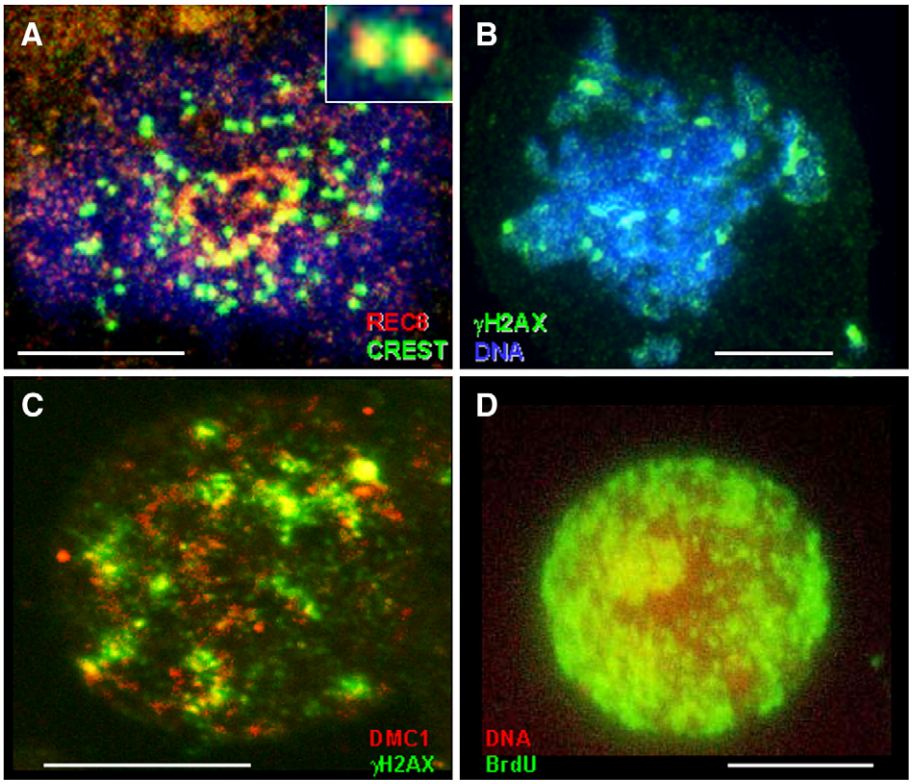

Fig. 2 – (A) IF of REC8 (red) and kinetochore (green) co-staining on a day 5 post IR ETC showing REC8 signals at kinetochores (insert) and a ring-like REC8 structure in this pro-metaphase cell. (B) Endo-metaphase ETC day 5 post IR displaying γ-H2AX-positive (green) chromosome regions (DNA: blue, DAPI). (C) DMC1 (red) and γ-H2AX (green) positive chromatin patches of a WI-L2-NS ETC, 5 days post IR. Mixed colour marks colocalisation. (D) BrdU labelling (green, 13 h pulse) of an ETC day 5 post IR indicating DNA replication. DNA is stained with Propidium iodide, red. Bars = 10 μm.

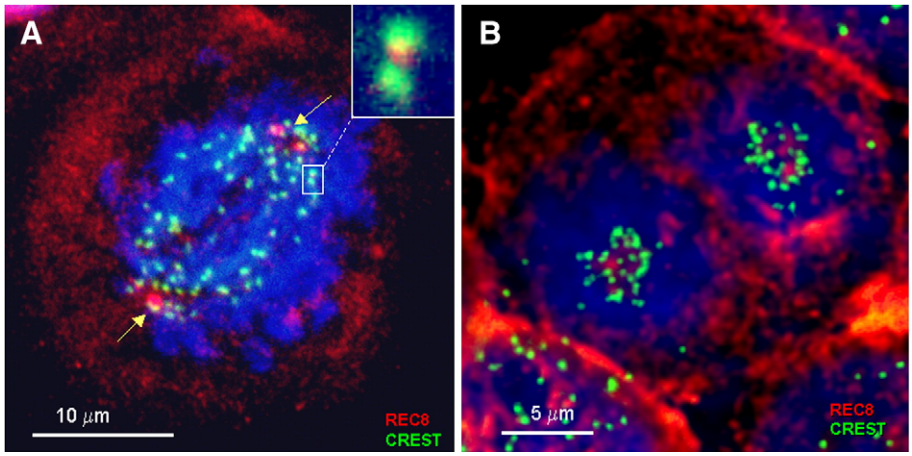

Fig. 3 – (A) REC8 (red) marks the spindle poles in an early anaphase of a Namalwa cell (day 5 post IR). Centromeres show only weak REC8 signals, with REC8 locating between some kinetochore pairs being a typical feature (boxed and enlarged on insert). (B) Telophase cell with REC8 (red) at the spindle poles identified by accumulated kinetochores (green dots). (B) — sampled after 2 h treatment with calpain inhibitor. Confocal images. Bar = 10 μm.

Altogether, these data show that REC8 dynamically localises to centromeres and spindle poles in ETCs. While the centromere localisation of REC8 may play a role in the cosegregation of sister chromatids (see below), its role in ploidy reduction of ETCs remains to be explored.

Western blotting of REC8

To better understand the role of REC8 in the divisions of ETCs we assessed its expression by Western blotting. Previously we had observed with anti-REC8 antibody sc-15152 (Table 2) a REC8-positive band of ~95 kDa [26]. Here, using a different anti-REC8 polyclonal antibody (Proteintech 10793-1-AP, Table 2) we detected two major bands of ~80 and ~95 kDa (Fig. 4). Treatment with alkaline phosphatase (AP) revealed a faint third band at about 70 kDa in untreated cell extracts in both tumour cell lines examined (Fig. 4). The results on the presence of high molecular weight REC8 bands agree with similar observations in rat meiocyte preparations [28] and in the human erythroblastoid leukemia cell line K562 (www.ptglab.com). Inhibition of protein degradation by incubation with calpain inhibitor (CI), which was the least toxic among 3 tested (Mg-132, lactocystin, and inhibitor of calpain), did not significantly alter the Western blotting results in irradiated samples although a switch in the relative prevalence of the 80 kDa and 95 kDa form was observed in untreated cells. Irradiation caused the overall increased expression of REC8, as noted

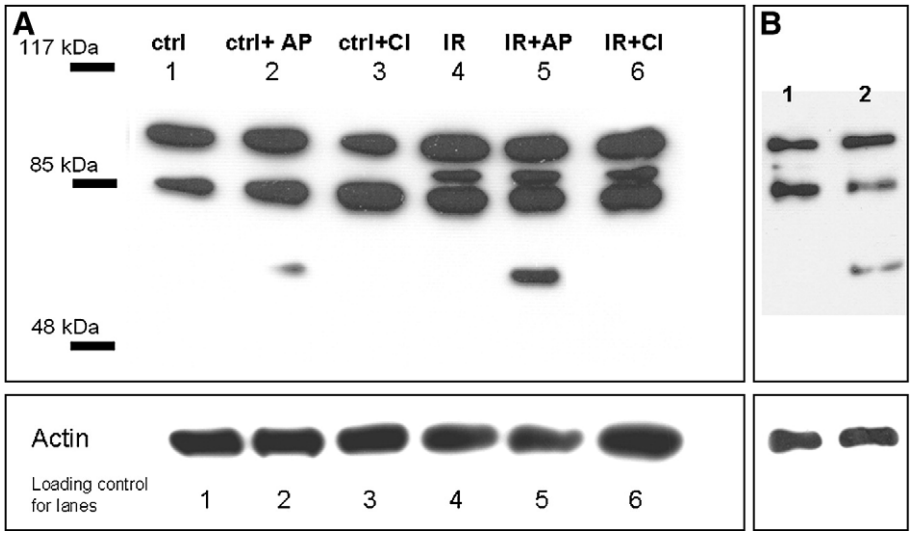

Fig. 4 – (A) REC8 immunoblotting of Namalwa cell lysates: in control (ctrl) and 5 days post 10 Gy IR (IR), after alkaline phosphatase (AP) and preincubation for 2 h with calpain protease inhibitor (CI). Lanes 1 — control; 2 — control (ctrl) + alkaline phosphatase (AP); 3 — control + calpain inhibitor (CI); 4 — IR; 5 — IR + AP; 6 — IR + CI. (B) REC8 immunoblotting of WI-L2-NS cell lysates: 1 — ctrl; 2 — ctrl + AP. (A) It is apparent that irradiation of Namalwa cells stabilises the 70 kDa and 90 kDa bands. Actin was used as a loading control.

previously [26], but in particular increased the expression of a fourth REC8 band of about 90 kDa which was seen only weakly in some extracts of non-irradiated cells (Fig. 4A) and in WI-L2-NS cells, while a 70 kDa protein species was observed only after AP treatment (Fig. 4). Since the non-modified form of REC8 has an expected Mw of 63 kDa it is likely that the ~70 kDa band represents the monomeric, non-phosphorylated form of REC8 and that the other forms of REC8 observed represent either post-translationally modified forms [28] or alternative transcript products [52]. Interestingly, the appearance/stabilisation of the 90 kDa band (Fig. 4), following irradiation indicates that this REC8 species/modification in particular can be upregulated by a radiation-induced process.

REC8 RNAi

To formally assess the function of REC8 on survival and ploidy reduction we attempted to generate cell lines with diminished *REC8* expression using RNAi technology. Although we were able to identify a number of siRNA sequences able to reduce the expression of REC8 in transient cell systems (Fig. S2), we failed to generate long term cell lines with reduced levels of REC8. To explore whether loss of REC8 might itself be deleterious we performed cloning efficiency experiments that revealed that cells transfected with the siRNA-REC8 plasmid were ~10 times less likely to survive selection (not shown). Clones that did survive, initially displayed down-regulation of *REC8* transcription as judged by qPCR in both Namalwa and WIL2NS cells but REC8 protein levels were hardly effected, particularly after radiation (data not shown). Therefore, although the RNAi approach was not particularly effective, the initial cloning efficiency experiments may indirectly suggest a pro-survival role of *REC8* expression for tumour cells.

ETCs display delayed DMC1/Rad51 repair foci

To further explore the survival/repair pathway in the ETCs we assessed the expression of γ-H2AX and Rad51 foci. On days 4 to 6 post IR a wave of cells expressing γ-H2AX domains and RAD51 foci was observed (Fig. S1B,a), in the agreement with previous observations [25]. This indicates delayed/secondarily induced DNA double-strand breaks (DSB) [43,53] and attempted DNA repair by homologous recombination (Table 1). Using co-staining for γ-H2AX and the meiotic recombinase DMC1 (with a DMC1-specific antibody, Table 2), we found large patches of γ-H2AX in endo-metaphases and in ETCs (Fig. 2B). The γ-H2AX-positive ETC chromatin patches surrounded and colocalised with DMC1-positive foci (Fig. 2C). The DMC1 foci also colocalised with Rad52 recombination protein (Fig. S1B,b). Interestingly, Ianzini et al. [8] reported the transcriptional upregulation of SPO11, and DMC1 foci formation in HeLa and MDA-MB435 cells 4 days post IR. Since secondary DSBs may be associated with DNA synthesis during the endo S-phase of these p53-defective ETCs, we investigated this hypothesis by pulsing the cells with BrdU. Following pulsing for

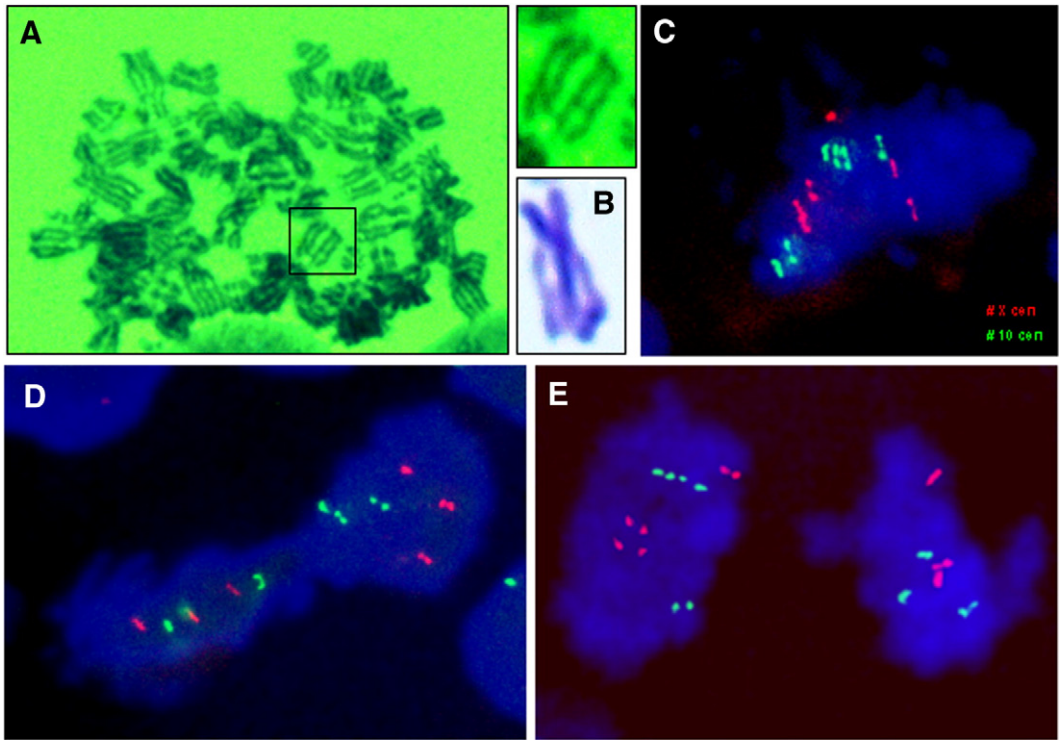

Fig. 5 – Chromosomes of (A, C–E) HeLa and (B) Namalwa cells. (A) DNA staining with Toluidine blue (A) reveals the tetranemic pattern of lengthwise aligned diplochromosomes (arrows) with a connection between the inner chromatids (boxed and enlarged) and (B) a cohesed pair of chromosomes (detail taken from a polyploid metaphase on day 5 post IR). (C–E) Irradiated cells after FISH staining for α-satellite sequences of chromosome #10 (green) and X (red). Centromeric target regions are forming three pairs of signal doublets in (C) early anaphase, (D) in anaphase, and (E) in telophase daughter chromosome masses, indicating the cosegregation of diplochromosomes.

1–2 h, these cells displayed large patches of BrdU-positive chromatin at 5 days post IR (Fig. 2D) suggesting that the DNA breaks detected by γ-H2AX and DMC1 staining (Figs. 2B, C) are associated with endoreduplication. Hence, both cytological and molecular features indicate that by the end of the polyploidisation phase (from day 4 post IR), a subset of the ETCs enter a process involving elements of the meiotic DSB repair response and that secondary breaks occurring during endoreduplication attract such DNA repair factors.

Segregation of diplochromosomes in ETC divisions

The staining patterns observed above suggest a potential role for REC8 in chromosome segregation in ETCs. Investigation of ETC metaphases from all tumour cell lines examined revealed the presence of diplochromosomes — chromosomes with connected sister chromatids seen as two binemic juxta-posed or lengthwise aligned chromosomes composed as tetrads (Fig. 5A). Often a bridge-like connection (Fig. 5A, insert) was seen between the inner chromatids of the pair and structures resembling crossovers between the two chromosomes were found in non-hypotonised samples after irradiation (Fig. 5B), with usually 1–2 such structures seen per cell.

To determine the segregation of sister chromatids (seen as kinetochore signal doublets) in dividing ETCs, we applied chromosome 10 and X-specific pericentromeric α-satellite repeat FISH probes to HeLa ETC preparations *in situ*. It was found that in ETC metaphase cells, centromere signals appeared as signal doublets (Fig. 5C), as is expected for replicated chromatids [54]. In early ETC anaphases the centromere-specific signal doublets segregated to opposite poles (Fig. 5D), instead of splitting up in single spots as expected in normal mitotic segregation. ETC sister centromeres still localised adjacent to each other in the separated chromosome masses of anaphase and telophase cells (Fig. 5E). Finally, there was a fraction of anaphase cells that contained normally segregated chromosomes with one signal for each centromere probe/chromosome mass (Fig. S1C). Approximately three times more ana-/telo-phases were seen with signal doublets than with normal centromere segregation.

The observations above suggest that segregation of diplochromosomes occurs in ETC anaphases, reminiscent of a feature of the reductional division in meiosis I.

BrdU labelling reveals unlabelled endopolyploid metaphase chromosomes

To determine, whether there are cells which omit S-phase between two mitoses in ETCs in the post irradiation time-course, we performed BrdU pulses for 13 h (not shown) and 20 h (each day from day 2 to day 10 post irradiation) using WI-L2-NS lymphoblastoid cells which have a cell cycle duration of about 17 h ([55] and Cragg et al., unpublished). Cells that fail to execute S-phase and progress to mitosis during a time frame longer than their normal cell cycle in the presence of BrdU will not incorporate it and produce non-labelled metaphase chromosomes in our assay. When we analysed Nocodazole-captured (2 h) metaphases cells ($n = 280$) and investigated their chromosomes for BrdU labelling patterns we found, besides the typical BrdU incorporation patterns, 9% and 2% of non-labelled metaphases at days 5 and 6 post IR, respectively (Fig. S3). These data suggest that the unlabelled metaphase cells failed to execute S-phase during pulse time (20 h), while still entering mitosis. This leaves, besides others, the possibility that some cells may enter mitosis without previous replication. Such an aberrant mitotic division has the potential to reduce genome size, a feature of ETCs that resume division [5]. Mitotic genome reduction division has also been noted in polyploid near-senescent fibroblasts [56]. Future research shall illuminate this possibility in more detail.

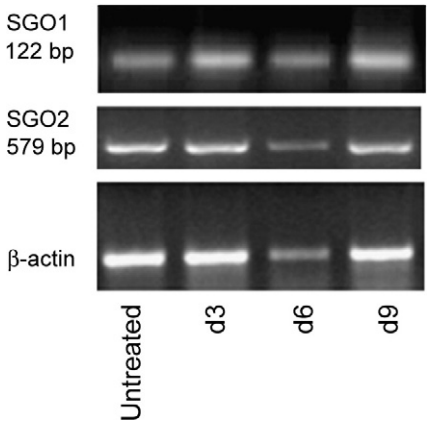

Fig. 6 – RT-PCR analysis showing the constitutive expression of SGOL1 (SGO1) and SGOL2 (SGO2) in irradiated tumour cells (A) without and at days 3, 6, 9 post 10 Gy acute irradiation.

ETCs express SGOL1 and SGOL2

During meiosis the release of sister chromatid cohesion is inhibited by Sgo1 which prevents the proteolytic cleavage of cohesin by separase on the chromosome arms at the onset of anaphase I through protection of REC8 [34,37]. A second human Shugoshin homologue, SGOL2, was found to be expressed in HeLa cells and seems to protect centromere spindle fibre attachment at metaphase (reviewed in Watanabe and Kitajima [32]). Since protection of centromere cohesion requires SGOL1 and spindle attachment SGOL2, we investigated whether SGOL1 and SGOL2 are expressed in ETCs. To this end, we performed RT-PCR for SGOL1 and sequenced the product to confirm its identity. This experiment showed that *SGO1* mRNA was expressed in Namalwa cells both before and post IR (Figs. 6 and S1D). Therefore, it seems likely that a REC8 containing cohesin complex involving SGOL1 could mediate sister centromere cohesion in ETCs that resume cell divisions >5 days post IR. SGOL2 was also expressed in the lymphoma cells both before and post IR, further supporting the observation that ETC express the capacity for meiotic-like ploidy reduction.

Such a mode of reductional segregation akin to that seen in meiosis I (see above) may contribute to genome reduction in the IR-induced ETCs thereby creating surviving tumour cells that escape radiation therapy.

Discussion

In the current study we have investigated p53-deficient tumour cells which undergo mitotic catastrophe and generate ETCs after irradiation. The resulting ETCs express REC8, a meiotic cohesin [29,31,33] that localises throughout the cytoplasm and in cytologically detectable amounts to interphase and metaphase kinetochores.

Furthermore, here we observed for the first time that REC8 was concentrated at the centrosome of interphase ECTs and at the spindle poles of giant ETCs undergoing bi- or multi-polar divisions. This particular sub-cellular localisation of REC8 was observed with two different antibodies and could be blocked with a competing REC8 peptide, indicating that the staining was specific. Furthermore, the spindle pole-associated REC8 colocalised with the microtubule-associated NuMA protein that is known to interact with the mitotic analogue of REC8 — SCC1/RAD21 and the cohesin complex. Cohesin in turn is required for the *in vitro* assembly of astral spindle poles and was found indispensable for division of HeLa cells [57].

Centromere cohesion in the meiosis I division involves protection of REC8 cohesin from separase action, which is mediated by shugoshin 1 [36]. Besides the REC8 cohesin our irradiated lymphoma cells were also found to express SGOL1 and SGOL2, as was the case for HeLa cells [32]. REC8's association with the ring-like NuMA-positive structures at prometaphase and to the metaphase/anaphase spindle poles may be mediated through a SMC1-dependent cohesin interaction with NuMa and Rae1, since the latter two proteins have been found to colocalise with spindle pole microtubules [58,59]. Taken together, our observations indicate that the ectopically expressed meiotic cohesin component REC8 associates with both centrosomes and kinetochores in IR-induced ETC.

These ETCs underwent bi-polar divisions wherein sister chromatids remained cohesed with each other and segregated as bi-chromatid chromosomes at anaphase. While arm cohesion is resolved in the first meiotic division, cohesion at sister centromeres is only released at the second meiotic division that lacks a preceding S-phase [27]. In the endopolyploid cells of our tumour cell line system it seems that cohesion at sister centromeres persists during this division leading to cosegregation of sister chromatids in some ETCs that resume division. This may occur only in a subset of cells, since most IR-induced ETCs undergo some form of cell death. Interestingly, cosegregation of sister chromatids, which is a feature of the meiosis I division, leads to the separation of homologs in some endopolyploid tumor cells. It thus seems that genome reduction in ETCs involves some features of both meiotic divisions, how this is exactly orchestrated remains to be seen.

In ETCs polyploidisation occurs through a series of endomitoses that increase the ploidy up to ~8–32C. Subsequently, a wave of cell death eliminates most such cells, while genome reduction seems to ensue in a subset. Interestingly, genome reduction by division has been noted in near-senescent polyploid fibroblasts [56]. Although the mode by which genome reduction is achieved in such fibroblasts or ETCs is currently mysterious, it's interesting to note that the ectopic expression and physiological localisation of meiotic gene products could be linked to the persistent sister chromatid cohesion observed in ETCs. This may involve REC8 in conjunction with SGOL1/SGOL2 expression, potentially leading to cosegregation of sister chromatids to opposite poles. Interestingly, irradiation induced an increase in the transcription and post-translational modification/stabilisation of REC8 as indicated by the appearance or stabilisation of a 90 kDa protein species in Western blots. Potentially, this induced form of REC8 could play a role in the observed events.

For homologs to be segregated they need to recognize each other and be physically linked, which in meiosis is mediated by recombinational DSB repair (reviewed in [38,48]). Interestingly, several cancer cell lines [8] and the ETCs shown here, express a number of meiosis-specific genes, among them the recombinase DMC1 [26]. In ETCs induced by irradiation, the expression of DMC1 peaks on days 4–6 post IR (Table 1). At this time a wave of delayed DSBs [53] and DNA repair by homologous recombination occurs in ETCs [43], which is corroborated by the presence of DMC1-positive foci colocalising with DSB-marking γ-H2AX foci [60] making it likely that secondary DSBs are involved. Delayed DSBs [53] and DNA repair by homologous recombination in ETCs [43] may be associated with DNA synthesis during the endo S-phase of ETCs as suggested by extensive BrdU incorporation 5 days post IR. Thus, the DNA breaks detected in these cells may be associated with endoreduplication and attract DNA repair proteins such as DMC1 and others. Interestingly, Ianzini et al. [8] have detected in HeLa, colon and breast cancer cell lines the radiation-induced activation of another meiosis-specific gene, *SPO11*, which is classically associated with meiosis initiation and physiological DSB formation (reviewed by Lichten [38]). Therefore, it will be interesting to investigate the activation and sub-cellular fate of this protein in ETC endocycles.

It is clear that ectopic DSBs could have the potential to contribute to the homolog recognition required for homolog segregation. Interestingly, it is already known that DNA damage can induce an association of homologous centromere regions in lymphocytes [61] a feature of DNA repair that could be used by ETCs to bring about homologous chromosome association. A possibility that is currently further explored.

Conclusions

We have observed that the meiotic cohesin REC8 is ectopically expressed in irradiation-induced ETCs where it localises to kinetochores and interphase centrosomes. In meta and anaphase ETCs, REC8 additionally localises to the spindle poles, suggesting a role of REC8 in the coordination of the endopolyploid centrosomal and chromosomal cycle. Altogether, it appears that the ETCs express features of meiotic divisions, albeit often in a disordered fashion, that could be important for the reduction of their genome size. Given its potential importance in regulating the survival and treatment-resistance of p53-defective tumour cells after genotoxic exposure, the molecular regulation of this peculiar somatic reductional division in ETCs deserves further investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi:10.1016/j.yexcr.2009.05.011.

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3.4 Up-regulation of the embryonic self-renewal

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Research Article

Up-regulation of the embryonic self-renewal network through reversible polyploidy in irradiated p53-mutant tumour cells

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ABSTRACT

We have previously documented that transient polyploidy is a potential cell survival strategy underlying the clonogenic re-growth of tumour cells after genotoxic treatment. In an attempt to better define this mechanism, we recently documented the key role of meiotic genes in regulating the DNA repair and return of the endopolyploid tumour cells (ETC) to diploidy through reduction divisions after irradiation. Here, we studied the role of the pluripotency and self-renewal stem cell genes *NANOG*, *OCT4* and *SOX2* in this polyploidy-dependent survival mechanism. In irradiation-resistant p53-mutated lymphoma cell-lines (Namalwa and WI-L2-NS) but not sensitive p53 wild-type counterparts (TK6), low background expression of *OCT4* and *NANOG* was up-regulated by ionising radiation with protein accumulation evident in ETC as detected by OCT4/DNA flow cytometry and immunofluorescence (IF). IF analysis also showed that the ETC generate PML bodies that appear to concentrate OCT4, NANOG and SOX2 proteins, which extend into complex nuclear networks. These polyploid tumour cells resist apoptosis, overcome cellular senescence and undergo bi- and multi-polar divisions transmitting the up-regulated OCT4, NANOG and SOX2 self-renewal cassette to their descendents. Altogether, our observations indicate that irradiation-induced ETC up-regulate key components of germ-line cells, which potentially facilitate survival and propagation of the tumour cell population.

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Abbreviations: ESC, embryonic stem cells; ETC, endopolyploid tumour cells; CSC, cancer stem cells; IF, immunofluorescence; WB, Western blotting; NT, non-treated cells; PIR, post-irradiation; ARS, accelerated replicative senescence; PBL, peripheral blood lymphocytes

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Introduction

Resistance to genotoxic treatments such as chemo- and radiotherapy, causes disease relapse and metastases in up to 50% of cancer cases. Recently, this property has been attributed to the presence of cancer stem cells (CSC) in the tumour population, which appear refractive to genotoxic treatment [1–4] or to 'stemloids' – proliferating tumour cells derived from progenitors which can reactivate self-renewal-capability [5]. Targeting these cells and their implied unique cell survival pathways is therefore an attractive proposition for cancer therapy.

The transcription network which tightly links self-renewal with pluripotency (the capability to produce all three germ layers) is regulated by three master genes *Oct4*, *Sox2* and *Nanog* [6,7]. Pluripotent stem cells derived from preimplantation embryos, primordial germ cells, embryonal carcinoma (teratocarcinoma) or artificially induced from somatic cells are unique in undergoing prolonged symmetrical self-renewal in culture. All of these pluripotent cell-types are capable of supporting embryonal development and express key embryonal stem cell (ESC) genes [6,8–10]. The pluripotency network has been found expressed in many types of adult stem cell [11] and in malignant somatic tumours correlating with poor clinical outcome [3,4,12]. However, interpretation of the data in somatic cells is currently complicated by the knowledge that OCT4 possesses several alternative splicing forms which can be regulated by multiple post-translational modifications and further by the fact that adult stem cells and somatic tumours express many pseudogenes of OCT4 and NANOG [13–16]. For example, those pseudogenes with similar open reading frames and high homology to paternal genes may mimic the activity of the homologous genes, or alternatively may act as their recombination templates, functional substitutes [17,18], or mRNA stabilisers [19].

In any case, the observation of a shared transcriptional program between pluripotent stem cells and tumours [20] provides the stimulus for investigating whether key pluripotency and self-renewal genes engender the basis for the treatment resistance of tumour cells (presumably through CSC or stemloids).

Evidence that endopolyploid cells induced after genotoxic treatment display increased resistance to DNA damaging agents and are involved in clonogenic survival has been now presented by several laboratories [21–24]. We have previously suggested the potential of this transient polyploidy to reactivate stem cell traits [25]. Here we asked if the core transcriptional cassette expressed by pluripotent stem cells is also expressed in somatic lymphomas and if it is up-regulated in the transient polyploid cells induced by genotoxic damage in p53-mutant tumour cell lines.

Materials and methods

Cell lines and irradiation procedure

The Burkitt's lymphoma cell line Namalwa was obtained from the American Type Culture Collection (ATCC) and has an established p53 mutant allele [26]. The human B-lymphoblastoid cell lines WI-L2-NS (*p53 mutant - mt*) and TK6 (p53 *wild type- wt*) were isolated from the same patient at different times during treatment and were obtained from Dr. P. Olive (Canada). Lymphoma cell-lines were maintained in RPMI-1640 containing 10% heat-inactivated fetal calf serum (FCS; Sigma) at 37 °C in a 5 % CO_2 humidified incubator. The EC cell line - human ovarian teratocarcinoma PA1 originated from the ATCC was obtained from the Vertebrate Cell Culture Collection of the Institute of Cytology (St-Petersburg, Russia). These cells were grown in DMEM medium containing 10% FCS (Sigma) and penicillin/streptomycin, and were harvested at sub-confluence as a positive control for ESC transcription factors [17,18]. IMR90 cells were obtained from ATCC (ATCC-CCL-186) and were grown in DMEM with 10% FCS for 30 passages, for the positive control of replicative cell senescence. For experimental studies, cells were maintained in log phase of growth for at least 24 hours prior to irradiation. Cells were irradiated with linear accelerator (Clinac 600 C, Varian Medical Systems) using a 4 MeV photon beam at a dose rate of 1 or 2 Gy/min. Correspondingly, a single acute dose of 5 or 10 Gy was delivered in all experiments. After irradiation, cells were maintained by replenishing culture medium every 48-72 h. In certain experiments, retinoic acid (Sigma; 1 μM) was added to the culture medium for 48 h from day 3 to day 5 post-irradiation. Smears of peripheral blood were used as a negative control for NANOG in immunofluorescence studies.

RT-PCR

Total RNA was extracted from cells by using TRIZOL (Invitrogen) and treated with DNase I (Fermentas MBI). cDNA was synthesized using First Strand cDNA Synthesis Kit (Fermentas MBI) according to the protocols of the manufacturer. The absence of contamination with chromosomal DNA was verified by PCR using primers *DQA-1* and *KIR3DL2*. Expression of pluripotent cell markers was verified in comparison to embryonal stem cells cDNA (Millipore, SCR063). cDNA from peripheral blood lymphocytes (PBL) was kindly provided by Dr. HTC Chan, Southampton University, UK.

Primers for PCR (Table 1) were designed using Primer 3 software [27]; the primers for OCT4 B1 isoform were taken in [28]. GenBank accession numbers of the templates used for design are as follows: OCT4-A (POU5F1), NM_002701; OCT4-B (POU5F1)_B, NM_203289; OCT4-B1 (POU5F1)_B1, EU518650; POU5F1B (POU5F1P1), NR_002304; OCT-4 ps4 (POU5F1P4), NC_000001; NANOG, NM_024865; SOX2, NM_003106. Amplification was carried out in a total volume of 50 μl with 1 – 4 μl cDNA in standard conditions using 0.5 units of *Taq* DNA polymerase (Fermentas MBI) with a BioCycler TC-S (BioSan). PCR conditions were as follows: 94 °C for 5 min (if not specified otherwise) 94 °C for 30 s, 56 – 60 °C for 20 s (for details see Table 1), 72 °C for 1 min; final extension step at 72 °C for 7 min. Amplified PCR products were resolved on 1.2 % agarose gels. The resulting PCR products fragments were analyzed by sequencing after ExoI/SAP treatment (Fermentas, MBI) using the fluorescent Big DyeTerminator v. 3.1 Cycle Sequencing protocol on a 3130xl Genetic Analyzer (Applied Biosystems).

Western blotting

For protein extraction, ProteoJET™ Cytoplasmic and Nuclear Protein Extraction Kit (Fermentas, Lithuania), along with Protease Inhibitor Cocktail (Sigma P8340) was used, according to the manufacturers' instructions. The purity of isolated cell nuclei was assessed by immunofluorescence using the antibodies against lamin B and α-tubulin (Fig. S1). Equal protein loading in each lane was checked by Ponceau S staining. As loading and fraction purity controls antibodies against GAPDH were used (positive staining

Table 1 – Sequences of primers, length of fragments, and annealing temperatures used for RT-PCR.

Gene/primer	Forward primer sequence 5'→3'	Reverse primer sequence 5'→3'	Length of fragment, bp	Anneal. temp., °C
β-ACTIN	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	234/329	58
DQA-1	GTGCTGCAGGTGTAAACTTGTACCAG	CACGGATCCGGTAGCAGCGGTAGAGTTG	225	56
KIR3DL2	CGGTCCCTTGATGCCTGT	GACCACACGCAGGGCAG	1941/369	56
NANOG	CACCTACCTACCCCAGCCTT	CTCGCTGATTAGGCTCCAAC	585	60
OCT4-A – AF/AR	TTCTCGCCCCCTCCAGGT	TCAGAGCCTGGCCCAACC	137	56
OCT4-B/B1 – BF/BR	GAAGTTAGGTGGGCAGCTTG	AATAGAACCCCCAGGGTGAG	267	56
OCT4-B/B1 – BF1/BR2	AGACTATTCCTTGGGGCCACAC	CTCAAAGCGGCAGATGGTCG	267/492	64
POU5F1B (*OCT4*-ps1)	AGGCCGATGTGGGGCTCAT	CCAGAGTGATGACGGAGACT	567	58
OCT4-ps4 – AF1/BR	AGGTTGGAGTGGGGCTAGTG	AATAGAACCCCCAGGGTGAG	289	56
SOX2	ACCTACATGAACGGCTCGC	CCGGGGAGATACATGCTGA	197	56

was observed in cytoplasmic but not nuclear lysates). Protein samples (10 µg) were separated by SDS PAGE on 9% gels and then blotted onto nitrocellulose membranes (Bio-Rad Labs). The membranes were blocked with 5% non-fat dry milk in PBS with 0.05% Tween 20 (Sigma) and primary antibodies applied overnight. The structure of OCT4 splicing isoforms and location of the epitopes for the two OCT4 antibodies used is presented in Fig. 1. The source and dilutions of antibodies is presented in Table 2 in the supplementary material. To confirm specificity, a blocking peptide for the OCT4 rabbit polyclonal (ab20650) was mixed with primary antibody at 1 µg/ml and incubated at room temperature (RT) for 30 min before application. Detection was performed with HRP-conjugated secondary antibodies (Table 2) and ECL (Amersham).

Two channel flow cytometry for OCT4 and DNA content

Cells were harvested at relevant time points, washed in cold TBS, and fixed overnight in cold (–20 °C) 70% ethanol. After two washes in TBS, cells were permeabilised with TBS/4% BSA/0.1% Triton X-100 for 10 min at RT. After that, samples were incubated with rabbit polyclonal anti-OCT4 antibody solution (5 µg/ml) (ab19857; Ab2, see on Fig. 1) in TBS/4% BSA/0.1% Triton X100 for 1 h at RT. Following three washes in TBS, cells were incubated with goat anti-rabbit Alexa Fluor 488 solution in TBS/4%BSA/0.1% Triton X100, 1:200 for 1 h in the dark. Then, DNA was counterstained with 10 µg/ml propidium iodide (PI) solution in PBS, containing 200 µg/ml RNAse (Sigma) and assessed by flow cytometry using a FACScan (BD Biosciences) using Cell Quest Pro Software.

Immunofluorescence (IF)

Standard IF staining was performed according to procedures detailed previously [29]. The primary and secondary antibodies used are listed in Table 2. Detection of apoptosis by FITC Annexin V (BD Pharmingen) in combination with IF was performed as detailed in [30].

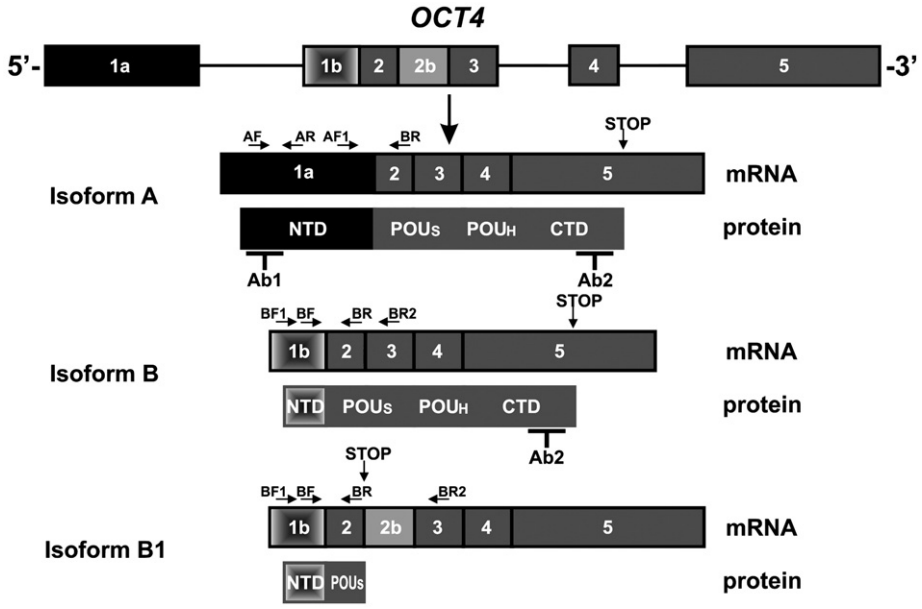

Fig. 1 – Structure of *OCT4*. Three different OCT4 isoforms are generated by alternative splicing which are schematically shown here with exons depicted as boxes. The RT-PCR primer binding sites and antibody binding epitope regions used in this study are indicated. POU_S – POU-specific domain, POU_H – POU homeodomain, NTD – N-transactivation domain, CTD – C-transactivation domain.

Table 2 – Antibodies: source and usage.

Primary antibodies:	*Secondary antibodies* (dilution, if not stated otherwise, 1:400)
Mouse monoclonal anti-hOCT4 C-10 (sc-5279, Santa Cruz) IF 1:100 WB 1:1000	Goat anti-mouse IgG-Alexa Fluor 488 (A31619, Invitrogen) or 594 (A31623, Invitrogen); Rabbit anti-mouse IgG-HRP (61-6520, Invitrogen) 1:4000
Rabbit polyclonal anti-hOCT4 (ab19857, Abcam) IF 1:75-400; Blocking peptide (ab20650, Abcam) WB 1:400-700	goat anti-rabbit- IgG-Alexa Fluor 488 (A31627, Invitrogen) or 594 (A31631, Invitrogen) Goat anti-rabbit IgG-HRP (32460, Thermo Fisher Scientific) 1:5000
Mouse monoclonal anti-hNANOG (N3038, Sigma) IF 1:50-75; WB 1:1000	Goat anti-mouse IgG-Alexa Fluor 488 or 594 (Invitrogen); Rabbit anti-mouse IgG-HRP (61-6520, Invitrogen) 1:4000
Rabbit polyclonal anti-hNANOG H-155 (sc-33759, Santa Cruz) IF 1:50-1:75; WB 1:400	Goat anti-rabbit- IgG-.Alexa Fluor 594 Goat anti-rabbit IgG-HRP (32460, Thermo Fisher Scientific) 1:5000
Rabbit polyclonal anti-hP16 N-20 (sc-467, Santa Cruz) IF 1:50	Goat anti-rabbit- IgG-.Alexa Fluor 594
Mouse monoclonal anti-hPML (sc-966, Santa Cruz) IF 1:150	Goat anti-mouse IgG-Alexa Fluor 488
Rabbit polyclonal anti-hAURORA B (ab2254, Abcam) IF 1:150-300	Goat anti-rabbit- IgG-.Alexa Fluor 594
Rabbit polyclonal anti-hSOX2 (sc-20088x, Santa Cruz) IF 1:500	Goat anti-rabbit- IgG-.Alexa Fluor 594
Mouse monoclonal anti-h-α-TUBULIN Clone B-512 (T5168, Sigma,) 1:2,000	Chicken anti-mouse IgG-FITC (sc-2989) 1:100
Goat polyclonal anti-hLAMIN B (sc-6216, Santa Cruz), IF 1:100	Rabbit anti-goat IgG-Cy3 (C2821, Sigma) 1:500
Mouse monoclonal anti-hGAPDH (MAB374, Millipore) WB 1:100	Rabbit anti-mouse IgG-HRP (61-6520, Invitrogen) 1:4000

Microscopy

A fluorescence light microscope (Leitz, Ergolux L03-10) in combination with a colour videocamera (Sony DX-S500) was used to examine cell preparations, record images, and perform image cytometry. For colocalisation studies a confocal microscope (Leica, DM 600), was used with the images scanned in the three different colour channels in sequence. Alternatively, the BRG filter system from Leica was used for the colocalisation calculations using a fluorescent light microscope (UV excitation band 385-415 nm, emission transmission band 455-475 nm; blue excitation band 487-503 nm, emission transmission band 515-545 nm; green excitation band 560-580 nm, emission transmission band 620-660 nm).

DNA image cytometry

DNA image cytometry was performed as detailed previously [31].

Clonogenicity assays

To measure clonogenic survival, a serial dilution assay was performed, using a method detailed previously [21]. Experiments were repeated at least three times.

Results

To assess the expression and induction of self-renewal genes in tumour cells before and after genotoxic treatment we performed experiments on three different lymphoma cell lines; Namalwa, WI-L2-NS and TK6. As we have published previously, the p53 mutant cell-line Namalwa is radioresistant, whilst the p53 wild-type TK6 is radiosensitive (Fig. S2A). WI-L2-NS (a *p53* mutant counterpart of TK6 derived from the same patient) is also radioresistant and displays 10-fold more polyploidy and two-fold less apoptosis than the *wt p53* TK6 cells in response to irradiation (Fig. S2B). Below we will detail the expression of the self-renewal genes first in untreated cells and then in cells after irradiation.

RT-PCR studies indicate the expression of pluripotency and self-renewal genes in lymphoma cell lines

OCT4 (POU5F) is a member of the POU family of homeodomain transcription factors required for the maintenance of pluripotency which is apparently achieved by establishing a transcriptionally permissive chromatin structure in the 160 kb *NANOG* locus [32]. Three splicing variants of *OCT4* gene have been hitherto described (Fig. 1) with OCT4A the classical form active in transactivation, OCT4 B a cytoplasmic isoform with unknown function [33,34] or possible function in cell stress of its alternatively translated variant [35] and the recently discovered OCT4 B1 splicing isoform [28] a potential additional marker of stemness [36]. Of seven OCT4 pseudogenes, the 1st (*POU5FB1)* has been recently confirmed as a transcriptional activator similar to *POU5F* [37], while the 3rd and 4th pseudogenes also have considerable open reading frames with transactivation potential.

Untreated cells

To assess the expression of *OCT4*, we performed semi-quantitative RT-PCR on the three lymphoma cell-lines before and after irradiation using a carefully designed selection of primers (for details see Materials and Methods, Fig. 1, and Table 1) and with the teratocarcinoma PA-1 as a positive control. Of the OCT4 splicing isoforms a transcript for OCT4A was found in all lymphoma cell lines and PA1 (Fig. 2A) and its identity was confirmed by sequence analysis. Expression of OCT4A was also confirmed in cDNA from bone fide ESC cells (Fig. S3 in Supplementary material). The OCT4 B/B1 isoforms were also found in all cell lines (Fig. 2A). The OCT4 B1 isoform, when detected separately from the B-isoform was found transcribed in PA1 and lymphoma cell lines. The OCT4B isoform was revealed in WI-L2-NS and TK6 cells at a higher level than PA1 cells. In Namalwa cells the expression of OCT4B was extremely low (Fig. 2A), but was confirmed by sequence analysis of the PCR product. Equivalent expression of the weak transcription activator *POU5F1B* (previously known as *OCT4*-ps1) was found in the lymphoma and PA1 cell lines (Fig. 2A).

Perhaps surprisingly, our RT-PCR analysis using primer AF1+BR (see on Fig. 1 and in Table 1) also revealed the presence

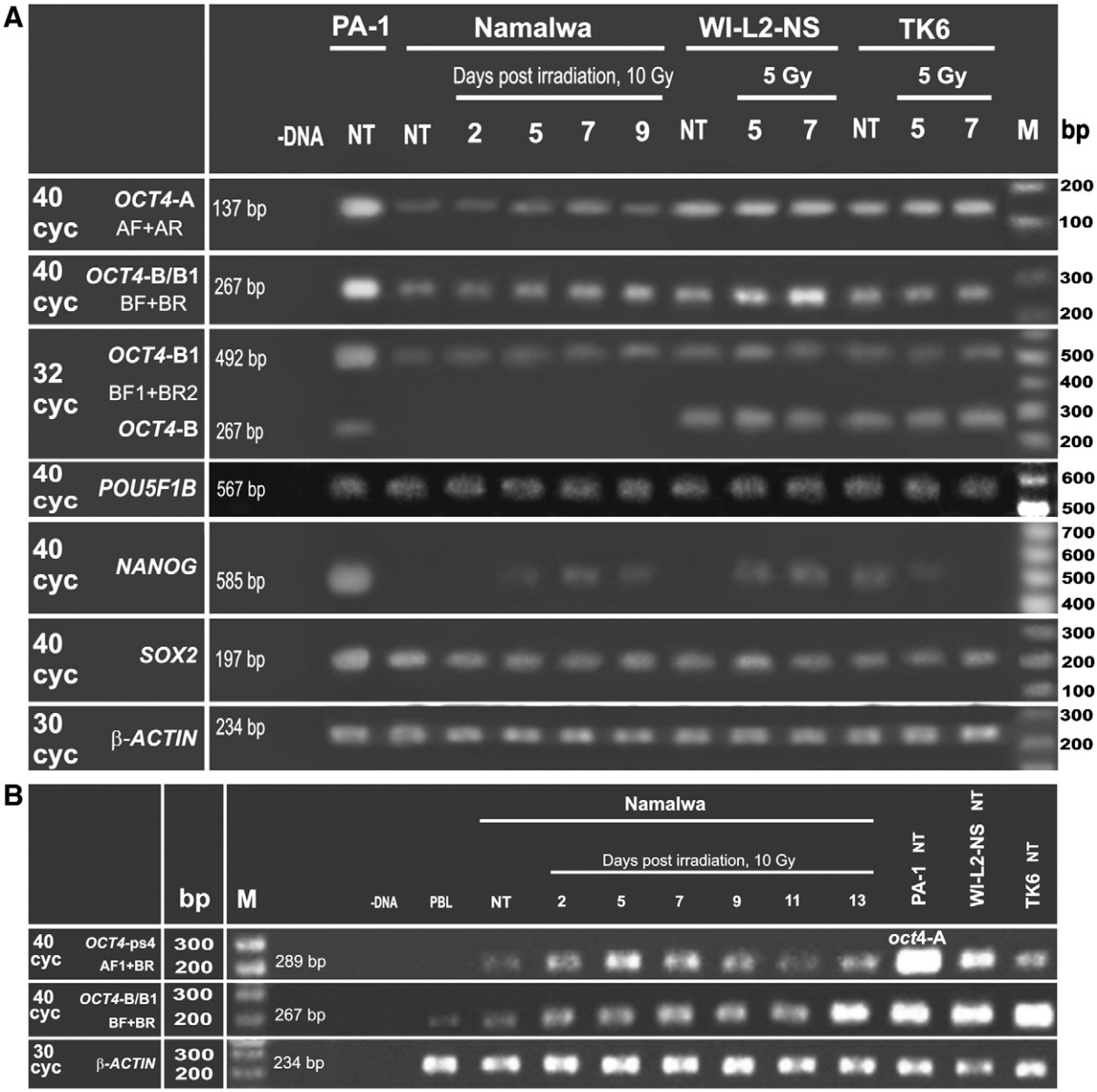

Fig. 2 – Semi-quantitative RT-PCR analysis. Untreated cells or cells treated with irradiation were assessed by RT-PCR. For primers, see Table 1. (A) Analysis for different *OCT4* (*POU5F*) isoforms, *POU5F1B, NANOG, and SOX2* showing their expression and dynamic changes in the post-irradiation time-course in three lymphoma cells lines. Non-treated PA1 cells (ovarian teratocarcinoma) were used as a positive control, while β-actin was used as an internal loading control. For location of primers in relation to *OCT4* structure, see Fig. 1. Expression of OCT4 A and OCT4 B/B1-isoforms and *NANOG* increased in *mt* p53 cell lines, Namalwa and WI-L2-NS, while *NANOG* expression decreased in *wt* p53 TK6 cells, after irradiation. *OCT4-ps1* (*POU5FB1*) was found equally expressed in all samples. (B) Expression of *OCT4A, OCT4* pseudogene 4 (OCT4-ps4), and *OCT4 B/B1* isoforms. In the positive control sample, ovarian teratocarcinoma PA1, OCT4 A and OCT4 B/B1 isoforms are expressed. In the negative control, peripheral blood leukocytes (PBL), only the OCT4 B/B1 isoform is expressed. *OCT4-ps4* is not expressed in PA1 and PBL but is expressed in lymphoma cell lines, where also *OCT4* B/B1 is present. Differentiation between the *OCT4A* isoform and *OCT4-ps4* pseudogene transcripts using primers AF1+BR was performed by sequencing. *OCT4 B/B1* and *OCT4-ps4* show concordant up-regulation after irradiation in the Namalwa cell line.

of a transcribed OCT4 pseudogene OCT4-ps4, which is similar in structure to the Oct4A isoform, in Namalwa, WI-L2-NS and TK6 cells, but not PA1 cells, where this primer amplified only true OCT4A as determined by sequence analysis (Fig. 2B). Interestingly, in the wt p53 TK6 cells there was reduced expression of this pseudogene compared with Namalwa and WI-L2-NS cells, whilst PBL did not express OCT4-ps4 at all (Fig. 2B).

Together, these data demonstrate that two OCT4 genes (*POU5F* and *POU5F1B*), all three OCT4 isoforms, and a pseudogene (OCT4-ps4) are transcribed at a low level in the untreated lymphoma cell lines and that the proportion of these components varies in each cell-line, perhaps indicating an aberrant regulation.

Transcriptional regulation of *NANOG* occurs in the *NANOG* locus through cooperative interaction with the OCT4:SOX2 heterodimer

[7,38,39]. In turn, *NANOG* is a central player in this transcriptional network, which serves to maintain OCT4 levels, sustains pluripotency, facilitates multi-drug resistance, and promotes cytokine independent self-renewal [40–42]. At least eleven pseudogenes of *NANOG* [43] exist although their expression and function in human tumours are little explored. Intriguingly, at least one of them - a retrogene *NANOGP8* was previously found expressed in cancers [18]. Expression of *NANOG* but not retrogene *NANOGP8* has been reported for the ovarian teratocarcinoma PA1 [18], which we used here as a positive control.

To avoid competition for the *NANOG* primer by pseudogenes of *NANOG*, the annealing temperature with the primer was elevated (Table 1). Under these conditions, *NANOG* expression was evident in PA1 cells but not-detectable in non-treated (NT) Namalwa and WI-L2-NS and weak in TK6 cells (Fig. 2A). As expected, PCR using the conditions detailed above confirmed expression of *NANOG* in cDNA from ESC cells (Fig. S3 in Supplementary material).

SOX2, in conjunction with OCT4, stimulates its own transcription as well as the expression of a growing list of mutually targeted genes. The essential function of SOX2 is to stabilise ESC into a pluripotent state by maintaining the requisite level of OCT4 [7]. SOX2 appears to function as a molecular rheostat that controls the expression of a critical set of embryonic genes, as well as the self-renewal and differentiation of ESC [44]. The expression of *SOX2* was found here in all cell lines (Fig. 2A).

Expression post-irradiation

Following irradiation, the OCT4A isoform was up-regulated (Fig. 2A) 5-7 days later. The OCT4B/B1 isoform was also enhanced on days 5-9; with the B-isoform particularly notable for WI-L2-NS cells, whilst the B1 isoform was most prominent for Namalwa cells (Fig. 2A). NANOG was clearly enhanced on days 5-9 in the mt p53 cells, Namalwa and WI-L2-NS (verified by sequencing), whilst it became down-regulated in the wt p53 TK6 cell line (Fig. 2A). Post-irradiation changes in *SOX2* expression were not convincing and the expression of *POU51B* also remained unchanged (Fig. 2A). Interestingly, transcription of the *OCT4*-ps4 pseudogene was enhanced in Namalwa cells after irradiation similar to that for OCT4B1 (Fig. 2B).

In summary, *OCT4* and *SOX2* were shown to be expressed in untreated lymphoma cells and *OCT4A* and *NANOG* upregulated by irradiation in cells lacking wild-type p53 function. The transcription of these genes indicates the potential functionality of the pluripotency and self-renewal transcription network in the investigated lymphoma cell lines.

Western blotting reveals OCT4 and NANOG proteins in lymphoma cell lines

Western blotting using a specific monoclonal antibody directed to the first 134 amino-acids of the N-terminus of OCT4A (Ab1, for its OCT4 epitope see Fig. 1) revealed strong protein expression in PA1 cells (observed as a double band) in both nuclear and cytoplasmic fractions. Weaker expression was observed in similar Namalwa cell lysates (albeit as a single upper band), while the expression in WI-L2-NS and TK6 cells was very low (Fig. 3A). However, using a polyclonal antibody for the C-terminal end of OCT4, capable of binding both OCT4A and B-isoforms (Ab2), (for its OCT4 epitope see Fig. 1), OCT4 expression could be demonstrated in the nuclei of all three lymphoma cell lines (Fig. 3B). In this case, as above, the positive control PA1 cells displayed two bands in the nuclear fraction, while only the upper band was found in the lymphoma cells. It is interesting to note that similar results using the same antibody (Ab19857) were presented previously [16], with two OCT4 bands evident for an embryonal carcinoma (P19) and only a single upper band for neural stem cells, approximately at the same positions as in our gels. It should be noted that both anti-OCT4 antibodies would be able to react with the product of the *POU51B* gene whose transcription was detected in PA1 and lymphoma cell lines (Fig. 2A).

These Western blotting results support those obtained from the RT-PCR analysis that OCT4 gene products are expressed in lymphoma cells, albeit aberrantly. Importantly, the demonstration of *OCT4* protein products in the nuclear fraction suggests that they may possess transactivation function. In keeping with this assumption, the main down-stream target of OCT4, NANOG, was detected in the nuclear fraction of PA1 cells and was found up-regulated after irradiation in lymphoma cells (Fig. 3C).

Two channel flow cytometry for OCT4/DNA shows the up-regulation of OCT4 in endopolyploid tumour cells (ETC)

To confirm our findings regarding *OCT4* up-regulation and to assess the nature of the cells up-regulating its expression, two channel flow cytometry for OCT4 (Ab2, see on Fig. 1) and DNA content was performed on Namalwa cells before and after irradiation using PA1 cells as a positive control. In untreated Namalwa cells, a low level of OCT4, just above background was observed, which increased markedly after irradiation, (>50 % of Namalwa cells had up-regulated OCT4 as compared to the NT control by day 5) (Fig. 4A). Careful analysis of the data showed that whilst the level of OCT4 was equivalent in 2C and 4C fractions in

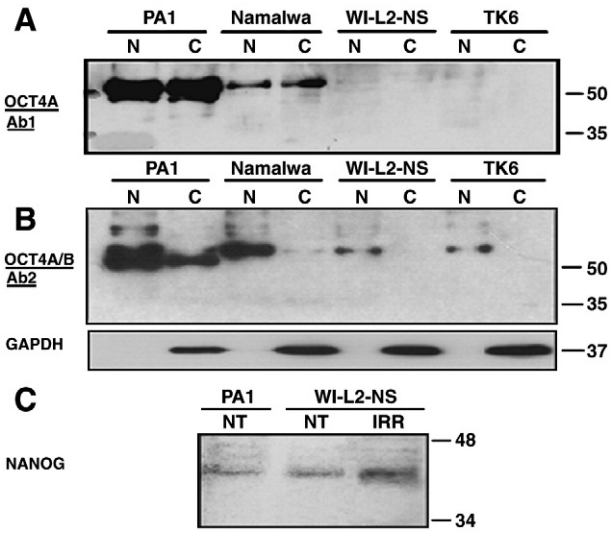

Fig. 3 – Western blot analysis of OCT4 and NANOG. Expression of the OCT4A isoform was assessed in PA1 and lymphoma cell line nuclei using the following antibodies: (A) Ab1 and (B) Ab2 with GAPDH also used as a fractionation control in (B). 'N' and 'C' designate nuclear and cytoplasmic fractions, correspondingly; (C) Expression of NANOG in the nuclear fraction of PA1 and WIL2NS cells. 'NT' – non-treated cells and 'IRR' – irradiated cells. For antibody sources and dilutions see Table 2.

untreated cells (Fig. 4B, upper panel), OCT4 expression was clearly elevated in higher ploidy (4C and 8C) fractions induced after irradiation (Fig. 4B, lower panel). The difference in OCT4 expression between NT and irradiated cells is further evident in Fig. 4C where the level of OCT4 positive cells in the 4C (2n, G2) component of NT cells is compared with the far higher level of OCT4 positive cells in the 4C (4n, G1) cells produced after irradiation. Previously we have shown that arrest in metaphase and mitotic slippage is the main method leading to polyploidisation of Namalwa and WI-L2-NS cells in response to 10 Gy irradiation [30,31,45]. In addition, here we illustrate DNA image cytometry data in WI-L2-NS cells (Fig. S2C) showing accumulation on day 5 after irradiation of metaphases with 4C, 8C and more DNA as well as a proportion of aneuploid (4C-8C) metaphases. Similar histograms were also obtained for irradiated Namalwa cells (data not shown).

Collectively, these results indicate that irradiated cells have shifted from the normal mitotic cycle to polyploidising (aborted mitotic) cycles up-regulating OCT4. Interestingly, the high level of OCT4 was also evident in the rare (~ 1.5%) polyploid cells observed in the untreated cell population detected by flow cytometry (Fig. 4B, upper panel) or IF (Fig. S 4a in Supplementary Material).

Immunofluorescence reveals the up-regulation of the pluripotency network in endopolyploid tumour cells (ETC) and their de-polyploidising descendants

ETC cells (>4C) comprise the major viable cell population on days 4-6 post-irradiation of p53 mutant lymphoma cells, whilst mitotic cycling of para-diploid cells is depressed. However, by the end of the first week after irradiation and secondary to a wave of apoptosis the polyploid survivors de-polyploidise and the growth of para-diploid mitotic cells is resumed [21,30]. To assess the expression of pluripotency and self-renewal transcription factors in these different cell populations we performed IF experiments using antibodies for OCT4, NANOG and SOX2.

OCT4 Ab1 (for epitopes see Fig. 1, Table 2) did not react with lymphoma cells, either NT or irradiated, however Ab2 (for epitopes see Fig. 1, Table 2) provided a weak positive reaction in NT lymphoma cell nuclei, which was enhanced post-irradiation in the ETC of WI-L2-NS and Namalwa, in line with the flow cytometry results. This increase in expression of OCT4 was accompanied by the enhanced expression of NANOG. To investigate any potential co-localisations, we subsequently combined staining with OCT4 Ab2 or the antibody for SOX2 with the monoclonal antibody for human

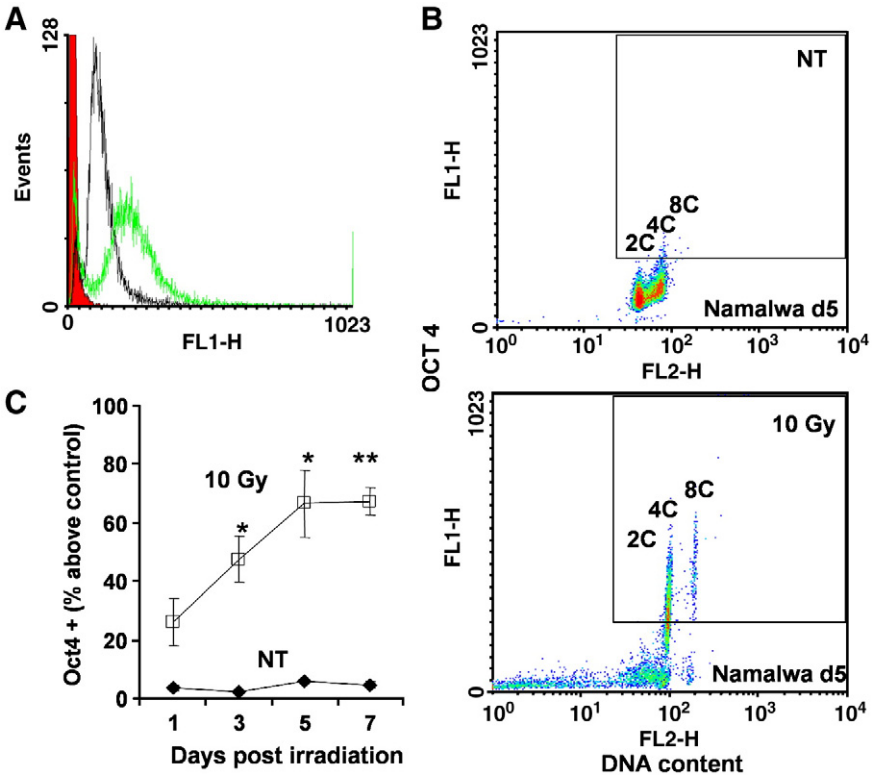

Fig. 4 – OCT4 protein expression in polyploid cells after irradiation. (A) Namalwa cells were irradiated and then on day 5 post irradiation cells were fixed, labeled with anti-OCT4 antibody (Ab2, see Table 2), and then assessed by flow cytometry. In comparison to NT cells (black line), an increased amount of OCT4 was observed in irradiated cells (green line). The solid red histogram represents background fluorescence omitting primary antibodies. (B) Two channel flow cytometry measuring DNA content (PI fluorescence) and OCT4 expression. In control samples, the level of OCT4 fluorescence was similar within the 2C and 4C fractions (upper graph). OCT4 expression was highly elevated in polyploidy 4C and 8C cells after irradiation (lower graph). (C) Kinetics of OCT4 fluorescence in 4C cells within seven days post irradiation. Cells were non-treated (NT) or irradiated, labeled with PI and anti-OCT4 antibody, and then assessed by flow cytometry as described. Data represent mean values of two independent experiments: S.E.M. * $p<0.05$, ** $p<0.007$. Two tailed Student t-test.

NANOG. These experiments revealed that both Namalwa and WI-L2-NS cells express low levels of OCT4/NANOG positivity in the nuclei of untreated cells (Figs. 5A, 6A). From day 3 post-irradiation ETC display enhanced nuclear staining for OCT4 with partial colocalisation with NANOG (Figs. 5 B, C). Furthermore, ETC positive for OCT4 staining were typically equivalently positive for NANOG.

As already reported, following polyploidisation, during the de-polyploidisation phase starting from day 5-6 onwards, the surviving ETC undergo bi- and multi-polar reduction divisions [29,31]. The nuclei of daughter cells resulting from these divisions are also OCT4- and NANOG-positive (Fig. 5D). Furthermore, during the early post-irradiation recovery period, the ETC are intensively dividing into para-diploid-sized cells which enter mitotic divisions symmetrically expressing in daughter cells the NANOG-bright cytoplasmic granules (Fig. 5E).

SOX2 staining of these same cell populations revealed similar enhanced expression in ETC, however it was more heterogeneous than for OCT4: different subpopulations of ETC showing on days 3-5 post-irradiation high and low intensities for SOX2, respectively (Fig. S 4b). In general, NANOG was up-regulated in the ETC with the moderately elevated levels of SOX2.

Notably, by 4 days post-irradiation, the enrichment of OCT4 was evident in the nuclei of almost all viable ETC of WI-L2-NS and Namalwa cells, with expression absent or greatly reduced in apoptotic ETCs as found by co-staining with DAPI or pre-incubation with Annexin V- FITC (Figs. S 4 c, d). In addition, both OCT4 and SOX2 were present in the centrosomal area of NT WI-L2-NS cells, often in the invagination into the nucleus (verified by co-staining for tubulin, Fig. S 4e). Moreover, these factors increasingly accumulated in this region in the treated ETCs (marked by asterix on Figs. 5C and 6A, B, H, I) and at astral poles in ETC undergoing bi- or multi-polar divisions (Fig. S 4f). OCT4 was also found in the centrosomal area of Namalwa ETC and was enhanced there by irradiation, although less prominently than in WI-L2-NS cells. This difference may be related to the much lower expression of the OCT4B isoform in Namalwa than WI-L2-NS cells as detected by RT-PCR.

In the positive control PA1 cells, Ab1 for OCT4 stained cell nuclei diffusely (Fig. S 5a), whilst Ab2 gave particulate and more heterogenous nuclear staining (Fig. S 5b), which was abolished by incubation with a specific blocking peptide (Fig. S 5c). The nuclei of PA1 also positively reacted with both antibodies for NANOG (Figs. S 5d, e). Peripheral blood lymphocytes were negative with both antibodies for NANOG (Figs. S5 f, g), however the cytoplasm of neutrophils reacted weakly with the monoclonal and more intensively, with the polyclonal antibody for NANOG (Figs. S 5 h, i), although the reasons for this were unclear.

The IF observations above confirm the up-regulation of key pluripotency and self-renewal transcription factors OCT4, NANOG and SOX2 in the ETC and their de-polyploidising descendants, which corresponds to the flow cytometry data and up-regulated transcription of the respective genes (and presumably functional pseudogenes) shown above.

Nuclear PML bodies integrate the transcription factors OCT4, SOX2, NANOG and initiate an intranuclear network in ETC

We subsequently studied the cytological mechanisms involved in the up-regulation of these self-renewal and pluripotency transcription

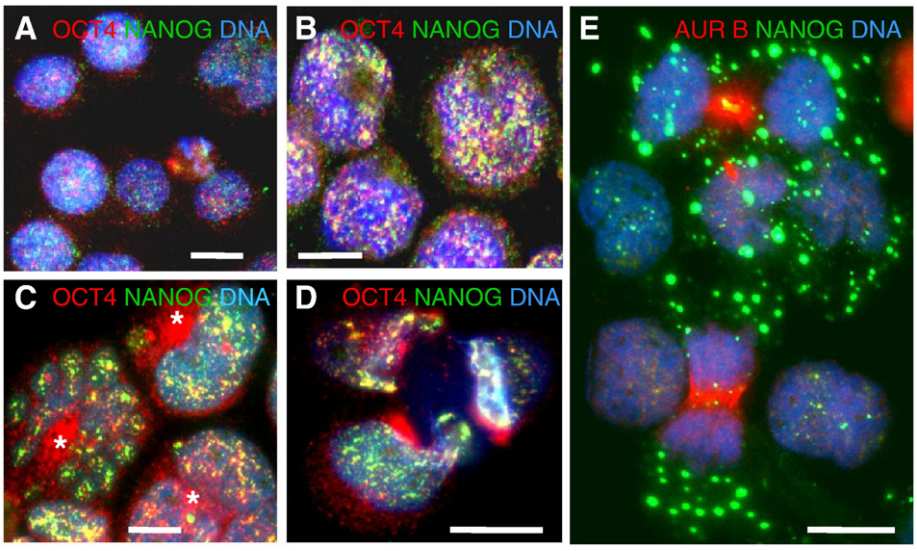

Fig. 5 – Expression and sub-cellular localisation of OCT4 and NANOG before or after irradiation. Namalwa or WI-L2-NS cells were non-treated (NT) or irradiated, pelleted, cytospined, fixed, and stained by the conventional IF procedure (detailed in [29]) for NANOG (monoclonal antibody) in combination with polyclonal antibody either for OCT4 (Ab2) or for AURORA B-kinase, DNA was counterstained with DAPI: (A) NT Namalwa cells showing background fine-speckled nuclear staining for transcription factors; (B, C) Namalwa and WI-L2-NS correspondingly, on day 4 after irradiation showing accumulation and partial colocalisation of both trascription factors in the nuclei of endopolyploid tumour cells (ETC); on (C) accumulation of OCT4 in the region of a centrosome is marked by asterix; (D) both transcription factors are still up-regulated in the nuclei of daughter cells resulting from tripolar mitosis of ETC on day 5 after irradiation of WI-L2-NS cells; (E) during extensive re-growth by bi-polar mitoses on day 7 after irradiation (mitotic spindles and mid-bodies are marked by AURORA B-kinase), the cytoplasm of daughter WI-L2-NS cells is symmetrically highlighted by bright NANOG-positive granules. Bars = 10 μm.

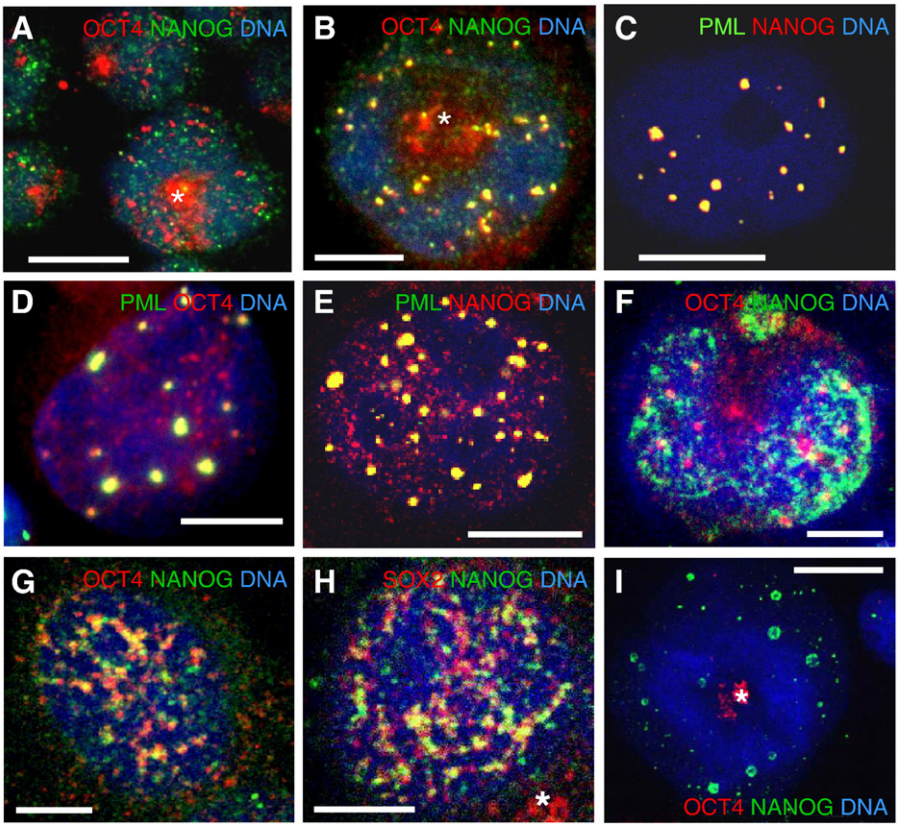

Fig. 6 – Sub-cellular localisation of nuclear PML bodies and pluripotency transcription factors in irradiated WI-L2-NS and Namalwa cells. WI-L2-NS (A-D, F, H, I) and Namalwa (E, G) cells were untreated (NT) or irradiated and then the expression and localization of OCT4, NANOG, SOX2 or PML bodies determined: (A) fine nuclear speckles of OCT4 and NANOG do not colocalise in NT cells; (B) however, begin to colocalise (often occurring as doublets) on day 3-4 after irradiation; (C) PML and NANOG colocalise in the nuclei of NT cells; (D, E) beginning of fusion between OCT4, NANOG, and PML in nuclear bodies, which increase in size, on days 4 after irradiation; (F) a rarer variant: NANOG partially colocalises with OCT4 positive nuclear bodies and is advanced further in the form of the intranuclear network on day 4 after irradiation; (G, H) alternative formation of the intranuclear network with increased clustering and more pronounced fusion of OCT4, SOX2, and NANOG on days 4-6 after irradiation. Fig. 6 H represents only one of three similar cell nuclei in a large ETC; (I) loss of nuclear localisation of the ring-shaped nuclear bodies (PML), full depletion of OCT4 and partial loss of NANOG from them, on day 5 after irradiation after 48 h incubation in the presence of retinoic acid (RA). The reactivity of centrosomes for OCT4 or SOX2 is marked on (A, B, H, I) with an asterix (*). Centrosomal OCT4 staining in the doughnut-like nucleus is retained on Fig. 6i after RA treatment. Antibodies: polyclonal (Ab2) – for OCT4 and monoclonal or polyclonal for NANOG (see Table 2). (C, E-I) represent overlays of confocal sections sequentially scanned in three colour channels through the inner part of the nucleus. Bars = 10 μm.

factors in ETC. The expression of these factors in untreated lymphoma cells was low, however small (0.2-0.5 μm) OCT4 and NANOG particles were detected, although they had very little association (Fig. 6A). However, within 3 days after irradiation, larger ~1.0-1.5 μm OCT4 granules appeared in the nuclei of emerging ETC, often as doublets and with increasing colocalisation with NANOG (Fig. 6B).

There is growing evidence that PML bodies represent dynamic sites for the assembly and post-translational modification of multi-protein complexes involved in various cellular responses including the response to DNA damage [46]. In particular, sumoylation at PML is necessary for the stabilisation, DNA binding, and transactivation potential of OCT4 [47]. Therefore we studied further a possible colocalisation of the induced OCT4 and NANOG nuclear granules with PML bodies. In NT cells significant colocalisation for NANOG with PML was found (Figs. 6C, 7), whilst OCT4 and SOX2 particles colocalised with PML bodies rarely in NT cells (Fig. 7). However, an increase in the number and size of PML bodies and their colocalisation with all three transcription factors was observed in irradiated cells, reaching saturation on day 4-5 post-irradiation (Fig. 6D, E; see counts on Fig. 7), coincident with the peak of polyploidisation [30]. At this time, the PML bodies apparently orchestrated the formation of extended chains of linked transcription factors promoting NANOG throughout the nucleus of ETC (Figs. 6F-H), with the distribution of SOX2 in relation to NANOG being very similar to that of OCT4 (Figs. 6G, H).

To assess the role of PML in integrating the pluripotency transcription factors, we used retinoic acid (RA) to down-regulate the activity of the *OCT4* promoter [48,49] and assessed its effects on the p53 mutant lymphoma cells. Addition of RA from day 3 to day 5 post-irradiation resulted in the impairment of the integrity and nuclear localisation of nuclear PML bodies, their loss of OCT4 and subsequent loss of NANOG expression (Fig. 6I).

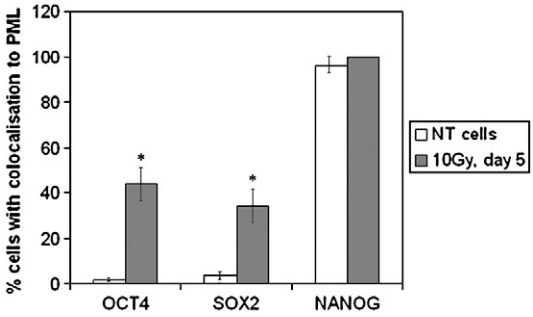

Fig. 7 – Colocalisation of pluripotency transcription factors with PML nuclear bodies. WI-L2-NS cells were left untreated (NT) or were irradiated and then on day 5 were fixed and stained for PML and either OCT4, SOX2 or NANOG plus DNA (DAPI) and imaged by colour videocamera attached to a fluorescent microscope through a BRG optical filter (for its optical characteristics, see Materials and Methods). Colocalisation of transcription factors (red) and PML (green) was considered positive if two or more foci per cell displayed yellow fluorescence. Damaged and apoptotic cells were not considered. Data represent mean values of two independent experiments: S.E.M. * $p < 0.05$. Two tailed Student t-test. Colocalisation of OCT4 and SOX2 to PML is found in 1-2% of NT cells, however increases up to 30-50% of irradiated cells, while NANOG is persistently colocalised with PML.

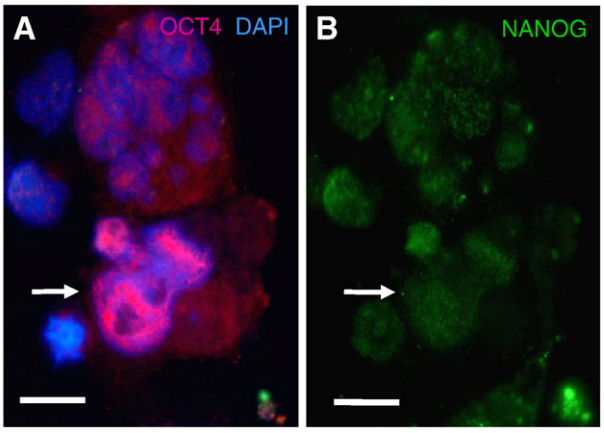

Fig. 8 – Lack of NANOG up-regulation in *wt* p53 tumour cells after irradiation. TK6 cells were irradiated and then on day 5 were fixed and stained for OCT4, NANOG and DAPI. The up-regulation of OCT4 in rare ETC of *wt* p53 TK6 cells was observed after irradiation (arrow on 'A') but was not accompanied by up-regulation of NANOG (arrow on 'B'), which becomes down-regulated in all TK6 cells.

The role of p53 in the self-renewal response of lymphoma cell lines

Contrary to the situation in p53 mutated cell lines, in p53 wild-type TK6 cells the response to irradiation was characterised by the loss of the initial nuclear NANOG staining within 3-5 days, even in rare giant cells expressing OCT4 (Fig. 8), while the p53-mutated counterpart WI-L2-NS displayed coherent up-regulation for both transcription factors in most ETC (as exemplified on Fig. 5). These data are in line with the down-regulation of NANOG transcription in TK6 cells presented above (Fig. 2).

These observations indicate that the key pluripotency and self-renewal transcription factors OCT4, SOX2 and NANOG are up-regulated through association with PML nuclear bodies and OCT4 and NANOG are co-ordinately expressed after irradiation in p53-mutated ETC. Further, our data indicate that this response to irradiation is impaired by functional p53 (in the radiosensitive lymphoblastoma cell line TK6) and by the suppressor of pluripotency, retinoic acid.

Antagonism between self-renewal and accelerated senescence in late ETC from p53-mutant lymphoma cell lines induced by irradiation

Mitotic catastrophe has been proposed as a cause of cell death either directly from arrested mitosis or indirectly through accelerated replicative senescence (ARS) with endopolyploidy as a hallmark feature [50,51]. In line with these data, senescence of a proportion of ETC produced after irradiation has been confirmed in our models previously starting from the end of the first week after irradiation as judged by the lack of BrdU inclusion, β-galactosidase-positivity and loss of the AURORA B-activity [31]. However, a smaller proportion of ETCs (~10% by day 14) continued to include BrdU and express CYCLIN B1 and AURORA B-kinase, indicating their escape from ARS [31]. Similar escape from ARS has also been described in lung cancer models [52,53]. Previously it was shown that in the absence of p53, cellular senescence is reversible [54] and that in this context p16 INKA 4a the negative regulator of the G1 to S transition serves as a second barrier preventing escape from cellular senescence [55]. Therefore, here we addressed the relationship between NANOG (as a marker of self-renewal and positive regulator of the G1 to S transition [42], p16INKA 4a (as a safeguard and marker of replicative cell senescence), and AURORA B-kinase (as a marker of cell division) in co-immunostaining experiments on irradiated WI-L2-NS cells. As reported above, we found low levels of NANOG in untreated cells, whilst p16 INKA 4a staining was negative (Fig. 9A). In the majority of ETC produced 3-5 days after irradiation OCT4 and NANOG were up-regulated in the mitotically potent (AURORA B-positive/p16 INKA 4a-negative) cells (Figs. 9B, C). However, routinely from day 6-7 after irradiation an increasing proportion of ETC began to down-regulate OCT4 and NANOG and many of them became p16 -positive (Fig. 9C). In parallel to this, a smaller cohort of ETC retained expression of self-renewal factors, continued DNA replication and mitotic activities up to de-polyploidisation, as already exemplified on Fig. 5D. Gradually, the p16 -positive ETC were cleared from cell suspensions (mostly by delayed apoptosis), although a small number were still evident on day 20 after irradiation when most of the cell population had been substituted by the new para-diploid cells (Fig. 9D).

These data clearly demonstrate that not all ETC become senescent but, on the contrary, indicate in line with our OCT4/DNA flow cytometry observations, that most ETC attempt to reactivate pluripotency and a considerable proportion of them retain high mitotic potential which appears to be directly supported by up-regulation of key members of the self-renewal program counteracting cellular senescence.

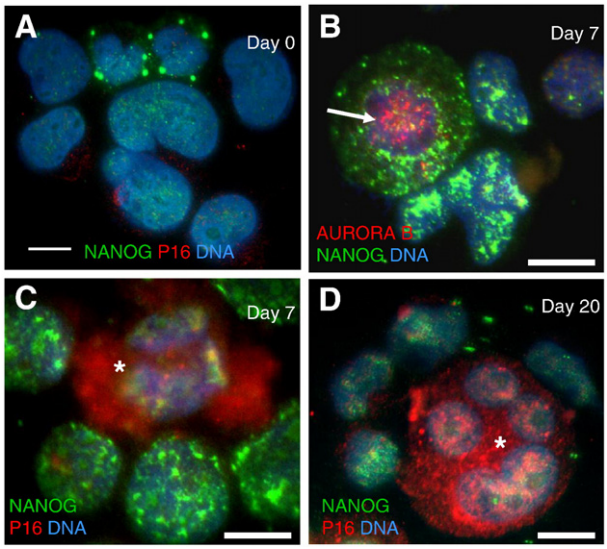

Fig. 9 – Antagonistic relationship between self-renewal and senescence markers in WI-L2-NS cells in the post-irradiation time-course. WI-L2-NS cells were left untreated or were irradiated and then at various times fixed and stained for NANOG, p16INKA 4a, AURORA B-kinase and DAPI. Samples in (A, C, D) are co-stained for NANOG and for the marker of replicative senescence p16INKA 4a. Sample in (B) was stained for NANOG and for the marker of cell division AURORA B-kinase, which localises to the centromeres of metaphase chromosomes of ETC rich with NANOG (arrow). Senescing ETC in (C) and (D) are marked by an asterix and up-regulate p16INKA 4a in the nuclei and cytoplasm and down-regulate NANOG. Bar = 10 μm.

Discussion

To assess the expression/up-regulation of key pluripotency and self-renewal genes in tumour cells we tested two different radioresistant p53 mutant B lymphoma cell lines before and after irradiation. Our detailed RT-PCR analysis provided data indicating that not only a recognised transactivator, OCT4A, but also two functional pseudogenes, an OCT4B cytoplasmic isoform and OCT4B1 may be involved in the response of lymphoma cell lines after irradiation. In agreement with recent reports [28], it appears that these varying components may be expressed aberrantly and differentially in each individual tumour cell line.

The current understanding of the interplay and function of the different OCT4 isoforms, including the functional pseudogenes is far from clarity [14,15,35,56], especially regarding their activities in somatic tumour cells [13,17,18,36,57]. Therefore, perhaps the most revealing of our findings concerned NANOG, a down-stream target and companion of the key pluripotency and self-renewal genes *OCT4* and *SOX2* [32]. Prior to irradiation, the basal level of *NANOG* transcription was low or un-detectable in both p53 mutant lymphoma cell-lines in keeping with published findings in haematological tumours [58,59]. However, we found by RT-PCR, IF and WB that *NANOG* became up-regulated after irradiation, indicating that the up-stream regulator genes *OCT4* and *SOX2* became functional upon irradiation in these somatic tumour cell-lines.

Importantly, our IF and flow cytometry observations revealed that the pathway involving these pluripotency transcription factors is up-regulated in lymphoma cells by genotoxic stress and for the first time show that this up-regulation occurs in ETC which are capable of undergoing de-polyploidisation transferring this phenotype to descendents. Although our data do not exclude the participation of OCT4 and NANOG pseudogenes in this response, several facts indicate that the induced ESC-like transactivation network is indeed regulated and functional: Firstly the co-localisation of NANOG with OCT4 and SOX2 in interphase nuclei of ETC and secondly their absence from mitotic chromosomes argue in favour of this conclusion. Up-regulation of the NANOG promoter is known to be associated with self-renewal symmetrical mitotic divisions [40–42]. Therefore, finding NANOG foci symmetrically distributed within the cytoplasm of ana-telophasic-early-G1 daughter cells recovering after DNA damage may be interpreted as a hallmark of such divisions, in continuity with the preceding de-polyploidisation of the ETC.

ETC, which were previously considered as reproductively inert, are becoming increasingly accepted as a possible survival alternative to cell death by mitotic catastrophe [23,60–62]. They emerge by abortive mitotic cycles and retain their proliferative potential as indicated by CYCLIN B1 and AURORA B-kinase positivity, the capability to recombine and repair DNA double-strand breaks involving meiotic proteins REC8, SGO1 and DMC1, and the ability to de-polyploidise [29–31,63]. Therefore, it is interesting to note that a similar reversible reproductive endo-polyploidy induced by mitotic catastrophe and associated with *OCT4*/*NANOG* expression was described for mouse and human ESC in vitro [64]. In this respect, it is also noteworthy that mixoploidy [65], genome instability [66], and the transcription of pseudogenes [67], are typical features of very early human embryo development and have direct parallels to the induced ETC investigated here.

The next question concerns how the key pluripotency and self-renewal transcriptional factors, which have only a background level of transcription in non-treated somatic tumour cells, become functional in ETC after genotoxic stress. In this regard, attention should be paid to the nucleation centres induced by irradiation, which appear to be initiated by OCT4 foci, expanding and fusing with SOX2 and NANOG in PML bodies. The association of these nucleation centres with the initiation of pluripotency was indirectly confirmed in experiments with retinoic acid, which caused loss of OCT4, NANOG, and nuclear localisation of these structures. As such, these OCT4-dependent nuclear foci appear to function as 'seeds' for the growth and extension of the NANOG-enriched nuclear network observed.

It is known that a certain threshold level of ESC factors is required to start the positive feed-back circuit of transcription activation [32,68]. Presumably, exactly these conditions are created in ETC emerging from p53 mutant cell lines after irradiation. In this situation, mitotic slippage leading to tetraploidy occurs and the G1 to S transition of tetraploid cells is favoured, providing an increased gene dosage of the key factors, which are further accumulating in the next polyploidising cycles, as many of these cells reach at least octaploidy ([21] and seen on Fig. S 2C). In addition, a cellular mechanism capable of bringing key pluripotency transcription factors physically together into nucleating centres supported and stabilised by PML bodies is induced. We further suggest that stabilisation of OCT4 along with PML-associated NANOG may favour looping out of the *NANOG*

chromatin domain to bring the *NANOG* promoter and other transcription factors together for interaction with Pol II as previously described [32]. Theoretically, this 'seeding' mechanism may also involve functional pseudogenes of OCT4 and NANOG transcribed from different chromosomes. The question of whether (and how) functional pseudogenes of OCT4 and NANOG are involved in the reactivation of the pluripotency state of somatic tumour cells remains a subject for further study. It would also be interesting to determine whether these ETC undergoing polyploidisation by abortive mitoses and de-polyploidisation by reduction divisions are biological candidates for the so-called 'stemloids' [5].

The self-renewal and plutipotency response described above apparently depends on inactivation of *p53* function as in p53 wild-type TK6 cells we observed the down-regulation of *NANOG* after irradiation in contrast to its induction in the p53 mutant counterpart WI-L2-NS. This behaviour is entirely anticipated as p53 is a well established down-regulator of the *NANOG* promoter [69] and also of endopolyploidy [70]. The radiosensitivity of the paired TK6 and WI-L2-NS cells was also previously shown to be primarily determined by the difference in p53 status of these two cell lines [71].

In our previous work [21,30] we studied the role of transient endopolyploidy as a survival alternative of mitotic catastrophe. Here, we extend these observations and indicate that self-renewal of lymphoma cell lines (probably both before and after irradiation) might occur by reactivation of a transcriptional programme similar to that engaged during early embryogenesis through activation of largely germ-line specific genes and their functional pseudogenes. This finding is in line with several recent reports in somatic tumours of various origins showing a correlation between chemo-radio-resistance and poor clinical outcome with the expression of OCT4, SOX2 and NANOG [3,4,12] or their down-stream targets of the pluripotency network [72].

In addition to the induction of the pluripotency and self-renewal genes, endopolyploidy induced by irradiation also causes activation of several meiotic genes including *MOS*, *REC8*, *SGO1*, *DMC1*, *SPO11* typically involved in metaphase arrest, genetic recombination, and reduction divisions [24,29,45,63]. These findings enabled us to previously suggest that this complex polyploidisation-de-polyploidisation response of tumour cells, extended for a week or more following genotoxic damage, possesses the features of an evolutionary asexual *life-cycle*, which transfers "stemness" from one generation of tumour cells to another [25,73]. Furthermore, Tam et al [74] have postulated that aged stem cells might be rejuvenated by reprogramming to an ESC-like state, while Curran et al [75] has shown that aspects of soma-to-germ-line transmission are important in *C. elegans* longevity. In line with these expectations, we have now confirmed that the reversible polyploidy does indeed involve induction of germ-line genes in ETC which counteract cellular senescence. This data provides a molecular explanation for the self-renewal and survival potential of tumour cells through reversible polyploidy.

Conclusions

In conclusion, our data show the clear up-regulation of the pluripotency and self-renewal transcription network in p53 mutant lymphoma cell lines induced by irradiation to undergo reversible endopolyploidy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.yexcr.2010.04.030.

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3.4.1 Possible participation of the splicing variant OCT4B (POU5F1)_B in the activation of lymphoma cells induced by irradiation

In normal human embryogenesis OCT4B is induced even before OCT4A isoform, but the function of OCT4B, which is mostly cytoplasmic protein remains unknown, however it is suggested to be involved in early stress response (Gao *et al.,* 2012). While studying response of lymphoma cells to irradiation we also applied a specific antibody to the N-terminal part of the OCT4B form. The study revealed accumulation of OCT4B at centrosome in the first days post-damage and further, the appearance from day 6 of OCT4B in the network of all three transcription factors (OCT4A, SOX2, NANOG), where it only partly colocalises with OCT4A (determined by Ab2) (Figure 9A). It is interesting that not only OCT4B but also meiotic proteins MOS (Vitale *et al.,* 2010 and our own unpublished observations) and REC8 were found in centrosomes of resting and dividing giant tumour cells (Figure 9 B, C). Centrosome is an integrator of the DNA damage response (Golan *et al.,* 2010). Therefore preliminary, this data suggests that OCT4B together with meiotic factors may participates in coordination of mitotic to meiotic transition through DNA damage checkpoint and induction of pluripotency in tumour cells.

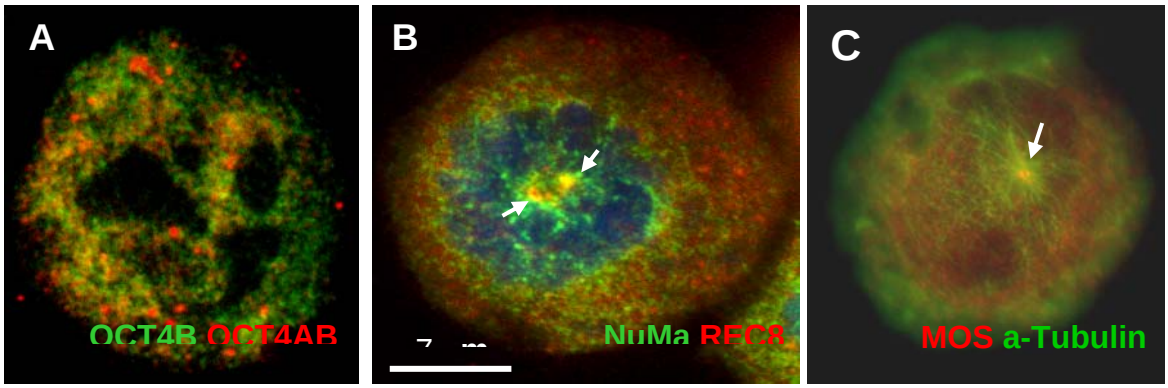

Figure 8. Centrosomal localisation of OCT4B, REC8 and MOS in endopolyploid tumour cells after irradiation (A) Partial colocalization of OCT4 antibodies (B) NuMa colocalization with REC8 in spindle pole of a ETC metaphase cell (arrow), (C) MOS forms a distinct signal at the centrosome marked by α-tubulin.

3.5. Polyploid tumour cells elicit paradiploid progeny through depolyploidizing divisions and regulated autophagic degradation

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Research Article

Polyploid tumour cells elicit paradiploid progeny through depolyploidizing divisions and regulated autophagic degradation

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Abstract

'Neosis' describes the process whereby p53 function-deficient tumour cells undergo self-renewal after genotoxic damage apparently via senescing ETCs (endopolyploid tumour cells). We previously reported that autophagic digestion and extrusion of DNA occurs in ETC and subsequently revealed that self-renewal transcription factors are also activated under these conditions. Here, we further studied this phenomenon in a range of cell lines after genotoxic damage induced by gamma irradiation, ETO (etoposide) or PXT (paclitaxel) treatment. These experiments revealed that chromatin degradation by autophagy was compatible with continuing mitotic activity in ETC. While the actively polyploidizing primary ETC produced early after genotoxic insult activated self-renewal factors throughout the polygenome, the secondary ETC restored after failed multipolar mitosis underwent subnuclei differentiation. As such, only a subset of subnuclei continued to express OCT4 and NANOG, while those lacking these factors stopped DNA replication and underwent degradation and elimination through autophagy. The surviving subnuclei sequestered nascent cytoplasm to form subcells, while being retained within the confines of the old ETC. Finally, the preformed paradiploid subcells became released from their linking chromosome bridges through autophagy and subsequently began cell divisions. These data show that 'neotic' ETC resulting from genotoxically damaged p53 function-deficient tumour cells develop through a heteronuclear system differentiating the polyploid genome into rejuvenated 'viable' subcells (which provide mitotically propagating paradiploid descendents) and subnuclei, which become degraded and eliminated by autophagy. The whole process reduces aneuploidy in descendants of ETC.

Keywords: autophagy; neosis; p53 mutant tumour; polyploidy; self-renewal; senescence

1. Introduction

Somatic polyploidy (endopolyploidy) and autophagy are paradoxically involved in execution of the opposing effects on tumour growth. While most ETC (endopolyploid tumour cells) induced after genotoxic or spindle damage of tumour cells lacking wild-type p53 function are lost for reproduction through mitotic death, apoptosis, necrosis or accelerated senescence (Ianzini and Mackey, 1997; Mackey and Ianzini, 2000; Shay and Roninson, 2004; Vakifahmetoglu et al., 2008), the capability of a small fraction of ETC to depolyploidize and provide clonogenic descendants has been repeatedly demonstrated (Baroja et al., 1998; Illidge et al., 2000; Sundaram et al., 2004; Puig et al., 2008; Ianzini et al. 2009; Vitale et al., 2010). In turn, although autophagy is a hallmark of cellular senescence (Kondo and Kondo, 2006; Vellai, 2009), it is activated upon acute induction of senescence (Galluzzi et al., 2009; Young et al., 2009) and may be oncosuppressive in certain contexts (Morselli et al., 2009; Galluzzi et al., 2010) or, when excessive, may accompany cell death (Scarlatti et al., 2009); both chemotherapy- and metabolic stress-induced activation of the autophagic pathway reportedly contributes to the survival of tumour cells (Edinger and Thompson, 2004; Debnath et al., 2005; Morselli et al., 2009; Galluzzi et al., 2010). In addition, autophagy extends organismal lifespan (Madeo et al., 2010).

Several years ago Sundaram, Rajaraman and colleagues (Sundaram et al., 2004; Rajaraman et al., 2006; Rajaraman et al., 2007) proposed the 'neosis' hypothesis to explain the rejuvenation and restoration of immortality through the descendents of supposedly senescing ETC based upon their interpretations of live cell imaging experiments. Subsequently, Tam et al. (2007) suggested that the reverse of cellular senescence may be associated with the capability of aging somatic cells to reactivate key embryonal proteins. Blagosklonny (2007) suggested that proliferating progenitors of cancer stem cells can be activated to become 'stemloids'. There are many indications in literature that cancer cells

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Abbreviations: ELCS, envelope-limited chromatin sheet; ETCs, endopolyploid tumour cells; ETO, etoposide; FCS, fetal calf serum; FMM, failed multipolar mitosis; IF, immunofluorescence; MDC, monodansylcadaverin; PXT, paclitaxel.

can overcome senescence (for example, Elmore et al., 2005; Roberson et al., 2005; Sabisz and Skladanowski, 2009). We recently (Salmina et al., 2010) found that up-regulation of embryonal self-renewal factors occurs in ETC induced after genotoxic insult and that this up-regulation is transmitted to the daughter cells upon depolyploidization. On the whole, up-regulation of pluripotency and self-renewal transcriptional factor NANOG was shown to antagonize activation of senescence regulator p16, and the latter further takes over only in a proportion of ETC (Salmina et al., 2010). These observations supported our previous finding that while a proportion of p53-deficient ETC activate senescence-associated β-galactosidase and down-regulate Aurora B-kinase, the other ETC continue its expression and division activities into the second week post-irradiation (Erenpreisa et al., 2008). Moreover, we and others also demonstrated (Erenpreisa et al., 2005; Kalejs et al., 2006; Ianzini et al., 2009; Erenpreisa et al., 2009; Vitale et al., 2010) that meiosis-specific genes and proteins are activated by stress-induced mitotic catastrophe and in resulting ETC, which are capable to undergo reduction divisions. Through live cell imaging, we further demonstrated that the progeny originated via depolyploidization retain proliferative capacity evidenced by their ability to produce colonies of mitotically dividing cells (Ianzini et al., 2009)

The central issue in this process of neosis is how depolyploidization and survival can be executed in a potentially mortal ETC, at the cellular and molecular level. Our first insight into the facet of this problem came over a decade ago where we demonstrated the dual activities of ETC in producing mitotic descendents and simultaneously undergoing partial chromatin degradation. In this latter process, a considerable portion of genetic material in viable ETC undergoes autophagic digestion and extrusion, indicating some form of sorting (Erenpreisa et al., 2000; commented by Wheatley, 2006). Further EM (electron microscopy) analysis showed that autolysosomes degrading the DNA are frequently induced in the cytoplasmic pockets of the nuclear ELCSs (envelope-limited chromatin sheets) (Erenpreisa et al., 2002), the structures whose role may be right in settling the chromosomes in the cell nuclei (Olins and Olins, 2009; Olins et al., 2011). Here, we extend our initial observations by revealing that different parts within the same multigenomic ETC can undergo degradation or self-renewal in order to give rise to viable clonogenic paradiploid survivors.

2. Materials and methods

2.1. Cell lines

Namalwa, Ramos and HeLa cell lines were obtained from the ATCC. The lymphoblastoma WI-L2-NS was obtained from Dr P. Olive (Canada). All cell lines are p53 function deficient. Lymphoma cell lines (Namalwa, Ramos, WI-L2-NS) were maintained in RPMI-1640 containing 10% heat-inactivated FCS (fetal calf serum; Sigma) at 37°C in a 5% CO_2 humidified incubator. HeLa S3 cells were grown in suspension under constant rotation in Joklik's MEM containing 10% heat-inactivated calf serum (Hyclone) and antibiotics. The adherent HeLa cells were cultured on glass cover slides in HAM-1 (Sigma) medium supplied with 10% FCS.

2.2. Cell treatments

For experimental studies, cells were maintained in log phase of growth and treated with a single acute 10 Gy dose of gamma irradiation (1–2 Gy/min, Clinac 600 C, Varian Medical Systems or using a Gulmay D3 225 X-ray source at a dose rate of 0.77 Gy/min), ETO (etoposide; Sigma) – 8 μM for 20 h or PXT (paclitaxel, Ebewe Pharma) – 50 nM for 20 h. Cell cultures were further fed every 2–3 days and sampled over a 2-week period posttreatment. To determine the capacity of cells to replicate DNA, BrdU was added at 5 μM to the cell culture for $\sim$24 h prior to fixation of cytospins with methanol as described previously (Erenpreisa et al., 2009).

2.3. Microscopy

A fluorescence light microscope (Leitz Ergolux L03-10) equipped with a colour videocamera (Sony DXC 390P) and a confocal laser microscope (Leica DM600) were used to capture fluorescent images. Phase contrast photography was performed using a light microscope (AxioImager A1, Carl Zeiss).

2.4. Cytological staining and DNA cytometry

For cytological studies, the cells were suspended in warm FCS and cytospun onto glass slides, fixed in cold ethanol/acetone (1:1) and air dried. Slides were hydrolysed with 5 N HCl for 30–60 s and stained with 0.05% Toluidine Blue in McIlvain 50% buffer pH 4–5 for 10 min, rinsed, dehydrated in warm butanol and xylene prior to embedding in DPX (Sigma–Aldrich).

To reveal NORs (nucleolar organizers), prefixed cytospins were stained with a 50% aqueous solution of $AgNO_3$ (Ural Factory of Chemical Reagents) diluted with 2% gelatin (2:1) at 60°C for 5–6 min and counterstained with 0.1% Methyl Green (Sigma–Aldrich) at room temperature for 1 min. This method is recognized as the universal indicator of NOR activity (Bancroft and Stevens, 1996).

Lysosomal activity and autophagic vacuoles were detected by staining for cathepsin B (see below) and with MDC (monodansylcadaverin, Sigma). For MDC staining, cell cultures were incubated with 0.05 mM MDC at 37°C for 1 h followed by fixation in 4% paraformaldehyde and two washes in PBS. The slides were counterstained with PI (propidium iodide; BD Biosciences Pharmingem), mounted into Permount (Thermo Fisher Scientific) and immediately imaged.

For conventional DNA staining, prefixed cytospins were hydrolysed with 5 N HCl at room temperature for 20 min and stained for 10 min with Toluidine Blue (pH 4.0). For DNA image analysis of mitotic cells where vacuoles were visualized in the cytoplasm, the hydrolysis with 5 N HCl was shortened to 30 s. DNA content was measured as the integral optical density in the green channel of the calibrated video camera using Image Pro Plus 4.1 software (Media Cybernetics; REO 2001). After shortened hydrolysis, interactive segmentation of mitotic figures was applied. Stoichiometry of DNA staining was verified using rat hepatocytes. With conventional 20-min acid hydrolysis, the 4C:2C ratio was 1.970; measurement error 1.5% (n=309); with 30-s hydrolysis, the 4C:2C ratio was 1.994; measurement error 0.3%

Table 1 **Antibodies: source and usage**

Primary antibodies:	Secondary antibodies (dilution, if not stated otherwise, 1:400)
Rabbit polyclonal anti-hOCT4 (ab19857, Abcam) 1:75–400 Blocking peptide (ab20650, Abcam)	Goat anti-rabbit-IgG Alexa Fluor 594 (A31631, Invitrogen)
Mouse monoclonal anti-hNANOG (N3038, Sigma) 1:50–75	Goat anti-mouse IgG-Alexa Fluor 488 (A31619, Invitrogen)
Rabbit polyclonal anti-hAurora B-kinase (Abcam, ab2254), 1:300	Goat anti-rabbit-IgG-Alexa Fluor 594 (A31631, Invitrogen)
Mouse monoclonal anti-hRAD51(ab213, Abcam) 1:100	Goat anti-mouse-IgG-Alexa Fluor 488 (A31619, Invitrogen)
Mouse monoclonal anti-h-β-TUBULIN (Neomarkers; clone DM1B) 1:100	Goat anti-mouse IgG-biotin antibody (1:100) (Vector Labs, UK) and by streptavidin-FITC antibody (1:150) (Vector Labs, UK
Mouse monoclonal anti-α-tubulin mouse (Sigma, B-512) 1:2000	Goat anti-mouse IgG-Alexa Fluor 488 (A31619, Invitrogen)
Rabbit polyclonal anti-g-H2AX (Trevigen; AMS Biotechnology, U.K. or 4411-PC-100, R&D Systems) 1:50	Goat anti-rabbit-IgG-Alexa Fluor 594 (A31631, Invitrogen)
Mouse monoclonal anti-bromodeoxyuridine (BdU) (A21300, Invitrogen) 1:200	Goat anti-mouse-IgG-Alexa Fluor 488 (A31619, Invitrogen)
Goat polyclonal anti-hRAD52 (C-17) (sc-7674, Santa Cruz) 1:50	Donkey anti-goat IgG-Alexa Fluor 488 (A11055, Invitrogen)
Rabbit polyclonal anti-hCATHEPSIN B (ab30443, Abcam) 1:100	Goat anti-rabbit-IgG-Alexa Fluor 594 (A31631, Invitrogen)

(n=182). Diploid DNA values for tumour cells determined after shortened hydrolysis were calculated by measuring metaphases and anaphase halves in 50 untreated cells, in which the variability coefficient was 7.7%.

2.5. IF (immunofluorescence)

Standard IF staining was performed according to procedures detailed previously (Erenpreisa et al., 2008). The primary and secondary antibodies used are given (Table 1). To detect protein disulfide isomerase, SelectFX Alexa Flour 488 Endoplasmic Reticulum Labeling Kit (S34200, Molecular Probes) was used according to the manufacturer's instructions.

3. Results

3.1. DNA-digesting autolysosomes appear in ETC late on after genotoxic and spindle damage

Following genotoxic insult, Ramos, Namalwa, and WI-L2-NS cells all undergo extensive polyploidization (Illidge et al., 2000; Ivanov et al., 2003), form ETC and subsequently begin chromatin extrusion coincident with the appearance of autolysosomes at the nucleo-cytoplasmic border as previously described (Erenpreisa et al., 2000, 2002). Many autophagic vacuoles could be stained for both DNA and for the lysosomal activation marker cathepsin B (Figure 1a) as well as for MDC, a marker of autophagic vacuoles (Figure 1b; for control, see Supplementary Figure S1 at http://www.cellbiolint.org/cbi/035/cbi0350687add.htm). The chromatin eliminated by autolysosomes was shown to be both TUNEL- (Erenpreisa et al., 2000) and γ-H2AX-positive (Figure 1c) indicating its selective nucleolytic degradation. The degradation and extrusion of large amounts of DNA from viable ETC was observed in lymphoma cell cultures after extensive cell loss through apoptosis or necrosis from the end of the first week postirradiation. In the control, untreated cells, polyploidy remained at low levels ($<$4%), and chromatin extrusion was extremely rare. These experiments were repeated on each lymphoma cell line at least six times with similar results.

A similar phenomenon was observed in HeLa cells at 6–14 days postirradiation (Figures 1d, 1e; data from one of four similar experiments) and at 4–6 days in WI-L2-NS and Namalwa cells after ETO and PXT treatments (exemplified in Figure 1f; data from one of three similar experiments). These data demonstrate that in these surviving ETCs, chromatin is sorted into two categories – one for preservation and the other for autodigestion and

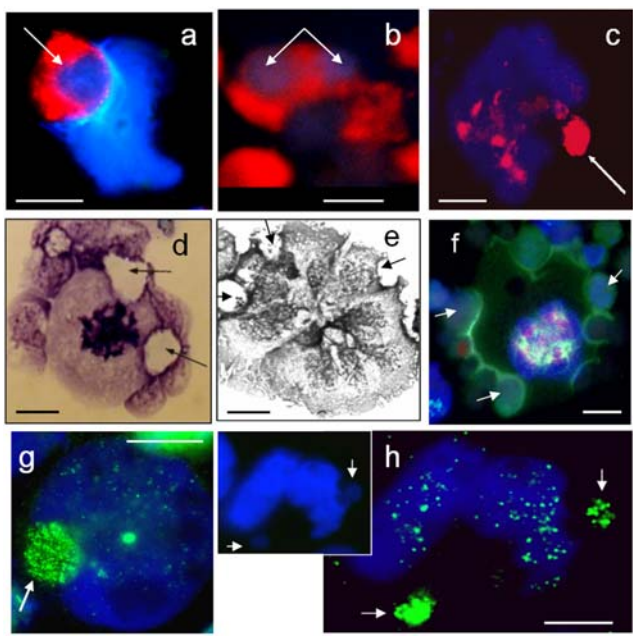

Figure 1 **Chromatin extrusion, mitotic activity and DNA repair in ETCs**
WIL-L2-NS ETC generated after irradiation (**a**) or ETO treatment (**b**) stained on day 8 with either DAPI (blue on '**a**') or propidium iodide (red on '**b**') to detect DNA and either cathepsin B (red on '**a**') or monodansylcadaverin (blue on '**b**'), as markers of autophagy. These data indicate that autolysosomes contain DNA. (**c**) Namalwa ETC induced by irradiation stained on day 5 for γ-H2AX (red) and DAPI, indicating the degradation of extruded chromatin (arrow); (**d**) HeLa ETC induced by irradiation stained on day 14 with Toluidine Blue (pH 5) after short acid hydrolysis, displays mitotic features and autophagic vacuoles (arrowed); (**e**) HeLa ETC stained with Toluidine Blue (pH 5) after short acid hydrolysis on day 9 after irradiation, reveals multipolar bridged anaphase and autophagic vacuoles (arrows); (**f**) WI-L2-NS ETC undergoing multipolar mitosis on day 5 after paclitaxel treatment, α-tubulin (green), Aurora B-kinase (red) and DNA (blue); multiple cytoplasmic 'bubbles' can be seen with destructured tubulin containing the DAPI-positive DNA remnants (arrows); (**g–h**) Namalwa ETC stained 6 days after irradiation for Rad 51 or Rad 52 (labelled in green) repair foci and extrusion of these recombinases are shown (arrows). In the inset to (**h**) only, the DAPI channel of the same cell is showed to indicate the location of DNA in the marginal vacuoles at the nucleo-cytoplasmic border (arrowed). The extruded chromatin is enriched with inactive DNA repair factors; DNA repair foci are evident in the other ETC depicted in the figure. Bars=10 μm.

elimination. Furthermore, it should be noted that the amounts of chromatin degraded can be considerable, for example, greater than 50% of a 16C ETC DNA content can be lost, and the whole subnuclei may be extruded in this way (Figure 1e, see also in Erenpreisa et al., 2000). As these cells possess the hallmarks of lysosomal and autophagic activity, often used as markers of senescence (Vellai, 2009), the question arises: are these cells senescing and entering irreversible growth arrest?

3.2. Compatibility of chromatin extrusion and mitotic activity in ETC

To address this question, we further analysed the ETC for signs of mitosis and chromatin extrusion. As shown, it is clear that ETCs are able to undertake mitotic activity (metaphase features in panel d; multipolar mitosis in panel e) (in Figures 1d–1e). This appearance was observed for all four cell lines. The question remains, however, as to whether these cells are also actively engaging in chromatin extrusion. Because of the proximity of what appears to be autophagic vacuoles (arrowed bodies in Figures 1d and 1e), it is tempting to speculate that the latter were extruded by the former. Although this conclusion can only be inferred; nevertheless, the features present (Figure 4e) would account for such interpretation. Thus, it is possible that chromatin extrusion may be compatible with mitotic activity in ETC and that the latter are not *bone fide* senescent cells (growth arrested by definition).

3.3. Chromatin extrusion in ETC is compatible with DNA repair and may participate in chromatin sorting

To address whether ETCs undergoing chromatin extrusion/degradation were capable of undergoing DNA repair, we stained ETC of Namalwa, HeLa and WI-L2-NS for the presence of DNA repair foci as labelled by the twin recombinases RAD 51 and RAD 52. These experiments were repeated for each cell line at least twice with similar results in each case. Again, because of the vicinity of the features present (Figures 1g, 1h), it is tempting to speculate that a portion of the ETC exerts both DNA repair and autophagic activities. Rad 51 staining appears in autolysosomal vacuoles as bundles of linearly arranged polymeric fibres (Figure 1g), possibly due to the loss of its proper association with Rad52 and DNA in the rejected chromatin (West, 2003). These observations imply a prior chromatin sorting process, which may occur during attempted DNA repair. It has been shown (Ivanov et al., 2003) that this repair occurs extensively in 40–80% of ETC before depolyploidization proceeds and requires suppression of p53 function; thus, we were further interested as to how these processes might relate to our recent findings regarding ETC and stem cell gene transcription (Salmina et al., 2010).

3.4. Diverse fate of sub-nuclei in late ETC

Most (~90%) ETC, which are formed by mitotic slippage from p53 function-deficient lymphoma cells after gamma irradiation begin to up-regulate the key germline transcription factors OCT4 and NANOG on days 3–5 postirradiation. These findings were observed in WI-L2-NS and Namalwa cells in greater than six experiments. In a similar fashion, HeLa cells displayed an up-regulation of these transcription factors (from a very low background level in untreated controls cells; data not shown), with an enhanced cytoplasmic expression (mostly in the centrosome) seen on days 4–5 postirradiation in most cells (Supplementary Figure S2a at http://www.cellbiolint.org/cbi/035/cbi0350687add.htm). However, nuclear up-regulation of OCT4/NANOG as exemplified (Supplementary Figure 2b) was found in only 5–10% of endopolyploid HeLa cells in three similar experiments.

Up-regulation was also found after treatment of WI-L2-NS cells with the radiomimetic ETO (not shown) and the stabilizer of microtubules PXT, where 98 and 85% of ETC, respectively, demonstrated a 2- to 3-fold increase of these germline transcription factors (typical staining is shown in Supplementary Figures 2c, 2d; representative of greater than three experiments). From day 5 to day 6 after these treatments, the surviving ETC of all cell lines initiated depolyploidization such that ETCs were found in bipolar and multipolar divisions, some after skipping S-phase (Erenpreisa et al., 2005, 2008, 2009; Ianzini et al., 2009). At these times, chromatin extrusion became observed. However, late on, large ETC displaying radial cleavage furrows during multipolar anaphase often do not complete cytotomy, and the restituted daughter nuclei assume a radial or horseshoe-like position in the ETC, while the furrows partly or completely regress (Figures 1e, 2; see also Erenpreisa et al., 2008, Figures 1d, 1e). This is a previously reported phenomenon, which is due to the hindrance of abscission by the chromosome bridges that exist between

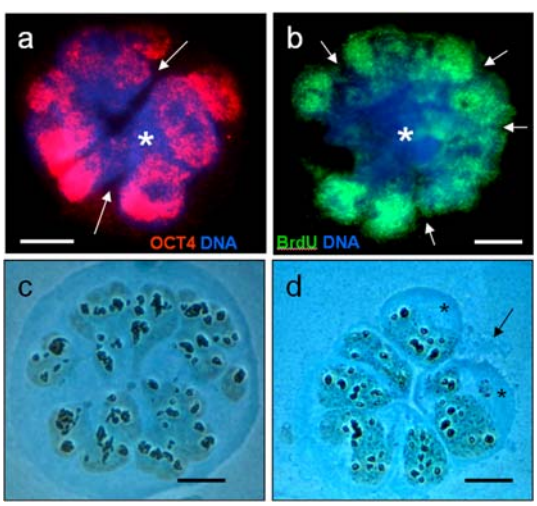

Figure 2 Differential rejuvenation and senescence of subnuclei in ETC after FMM

(**a**) Namalwa ETC stained on day 8 after irradiation for OCT4; the OCT4-positive subnuclei are found at the periphery of the ETC, while the OCT4-negative subnuclei occupy the centre (*); (**b**) BrdU uptake in Namalwa cells (following a 24 h pulse from day 7 to 8 after irradiation) revealed by immunofluorescence shows both replicatively active (green) and inert (*) chromatin within the ETC; (**c**, **d**) HeLa ETC observed on day 13 after irradiation stained for Ag-NORs, counterstained with Methyl Green and imaged in phase contrast. (**c**) Multinuclear ETC after FMM with five to seven subnuclei and still intact cytoplasm; (**d**) post-FMM ETC presenting degradation of the outer (old) cytoplasm and distribution of the inner cytoplasm enclosing the individual subnuclei (*); the remnant of the old cytoplasm is indicated by the arrow. Bars=10 μm.

subnuclei (Steigemann et al., 2009). In these ETC subnuclei, we see multiple Ag-positive NORs (Figures 2c, 2d), implying that these cells have recently passed mitosis and that the subnuclei have the capacity for further formation of ribosomes and ergastoplasm (see below). In late ~16C–32C ETC days 8–14 postirradiation, which, as judged by the radial cytoplasmic clefts and/or triangular-shaped nuclei, have undergone similar FMM (failed multipolar mitosis), we observed signs of the chromatin differentiation. Some subnuclei become rounded and separate from the remainder. They display markers of reproductive potential such as nuclear OCT4 (Figure 2a) and NANOG (data not shown) and replicate DNA (incorporating BrdU, Figure 2b). However, not all of the subnuclei stain positively for OCT4, and a region of (usually close to the centre) or even entire subnuclei are commonly OCT4 negative (Figure 2a, asterisk) and fail to include BrdU even in a 24-h incubation period (Figure 2b, asterisk). In our earlier pulse-chase experiments with ^{3}H-thymidine, we noted that the DNA in the extruded chromatin had been previously replicated in the ETC but had stopped replication prior to its expulsion from the cell (Erenpreisa et al., 2000). In practically all of the late ETC, derived from each cell line, we observed chromatin extrusion and the remaining vacuoles invaginating into the cell nuclei.

3.5. Diverse fate of cytoplasm parts in ETC

Next, we studied the fate of the ETC cytoplasm. We found that not only subnuclei but also the associated cytoplasm undergoes differentiation and separation in ETC undergoing FMM, whereby the external layer of cytoplasm becomes discarded, as the inner cytoplasm sequesters its territory around rejuvenated rounded subnuclei (compare Figures 2c and 2d). Furthermore, during the segregation of the cytoplasm, the nucleoli of rejuvenated cells become highly active, enriching the inner cytoplasm with ribosomes (Figures 3a, 3b); the rejuvenated subnuclei activate their surrounding ergastoplasm (Figure 3c) and organize the cytoskeleton with its own centrosome (Figure 3d). Thus, rejuvenated daughter subcells arise and become preformed within an old mother ETC prior to their release. This same phenomenon was observed in lymphoma cell lines and the HeLa cells.

3.6. Autophagy releases ETC daughter subcells from their constraining chromosome bridges

Depolyploidization of ETC is a poorly understood multistep process, where the multipolar divisions are suggested to play a role (Gisselsson et al., 2008; Vitale et al., 2010). As described before, ETCs undergo meiosis-like bi- and multipolar divisions (Erenpreisa et al., 2005, 2008, 2009; Ianzini et al., 2009); however the sequence, relationships and contribution of the various types of ETC divisions in the clonogenicity are currently far from clarity (Erenpreisa and Cragg, 2010). Here, we describe a particular facet of this complex process showing that after failed multipolar divisions, ETC sort and extrude some subnuclei, discard the original cytoplasm and preform rejuvenated daughter subcells with their own nascent cytoplasm. However, after failed multipolar mitosis, the chromosome bridges between daughter subcells are linked by the Aurora B-aided radial cleavage furrows into one or more knots (Figure 1e; 4a; also illustrated by Erenpreisa et al., 2005, Figures 1a, 1e, 1d; 2008, Figure 8a), preventing final abscission. As these structures do not persist, it appears that autophagolysosomes can engulf and digest not only the inert chromatin but also these knots. The formation of the autolysosome in the cell centre between preformed subcells is commonly observed during the disintegration of these ETC in all studied cell lines (Figure 4b). The process likely releases free energy leading to repulsion of daughter subcells (Figure 4c) and possibly even a flare of lysosomic autofluorescence (Figure 4d). The repulsion of the daughter subcells is reminiscent of budding (see also in Erenpreisa et al., 2005, Figure 1f). Importantly, the budding subcells are enriched with the self-renewal factors OCT4 and NANOG (Figures 4c, 4d) and resume cell divisions (Figures 4d–4f). The mitotic cells released from ETC mostly contain a paradiploid amount of DNA reducing aneuploidy characteristic for ETC as determined by interactive image cytometry on WI-L2-NS after ETO treatment (Figure 5). Broadly similar things happen in HeLa cells (data not shown).

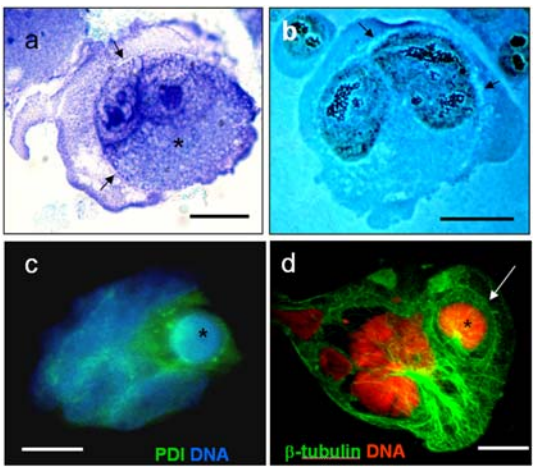

Figure 3 Differential rejuvenation and senescence of cytoplasm in ETC
(**a**) HeLa ETC on day 8 after irradiation stained with Toluidine Blue, pH 5, after a short acid hydrolysis; splitting off the outer layer of cytoplasm (the fissure is arrowed) from the inner area enriched in ribosomal RNA is visible (*); (**b**) HeLa ETC on day 8 after irradiation stained as on Figures 2c, 2d; nuclei are actively engaged in ribosomal synthesis (Ag-NOR positivity of the developed nucleolonema), while the outer cytoplasm is splitting from the inner (the fissure is arrowed); (**c**) WI-L2-NS ETC observed 8 days after ETO treatment, immunostained for the ergastoplasmic marker protein disulfide isomerase (PDI); the high activity of PDI (green) around single rounded subnuclei (asterisk) is evident. (**d**) Confocal microscopy image of Namalwa ETC observed 6 days after irradiation and stained for β-tubulin and DNA using propidium iodide; a separating subcell (*) can be seen within its own cytoskeletal network (arrow) distinct from the linked network containing several other subnuclei. The latter displays converging microtubular branches – possibly the result of incomplete multipolar mitosis. Bars=10 μm.

4. Discussion

There is a view that polyploidy may serve as a link between quite opposite biological processes as cellular senescence and self-renewal, thereby paving the road to cancer or its progression (Rajaraman et al., 2006; Mosieniak and Sikora, 2010). Here, we

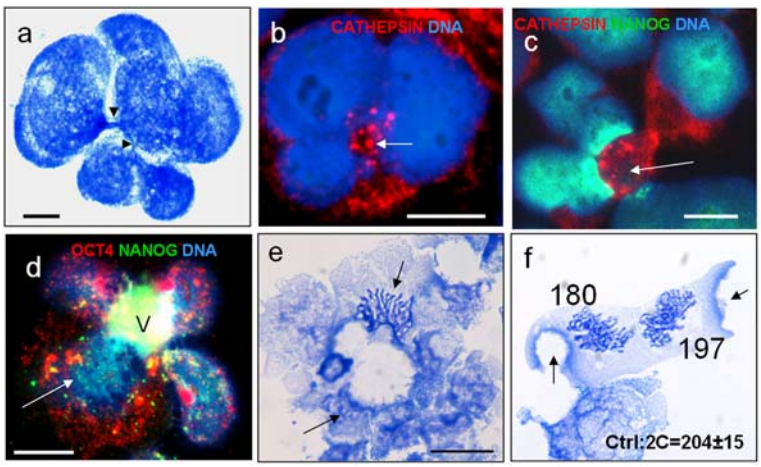

Figure 4 Potential role of autolysosomes in the final disintegration of ETC

(**a**) HeLa ETC on day 5 after irradiation stained with Toluidine Blue pH 5 after short acid hydrolysis; after incomplete multipolar mitosis, daughter subcells remain linked by a series of chromosome bridges (arrowheads). (**b**) Cells on day 9 after irradiation stained for cathepsin B and DNA; distinct cathepsin-positive foci can be seen in the central cytoplasmic area between subnuclei (arrow). (**c**) WI-L2-NS ETC on day 8 after irradiation stained for cathepsin B, DNA and NANOG; cathepsin-positive autolysosome (arrow) is apparently dissociating NANOG-positive subcells. (**d**) WI-L2-NS ETC stained on day 9 after irradiation for OCT4, NANOG and DNA (DAPI); a flare of autofluorescence is observed in the central autolysosome (V) possibly repulsing the OCT4/NANOG-positive subcells, one of which is in metaphase (arrow). (**e**) Namalwa cells on day 14 post-ETO treatment stained with Toluidine Blue pH 4 after shortened acid hydrolysis; separation of subcells by a central autophagic vacuole; the arrows indicate two separated anaphase halves. (**f**) WI-L2-NS cells on day 14 post-ETO treatment stained with Toluidine Blue pH 4 after shortened acid hydrolysis; a distinct ETC daughter cell in anaphase with the remnants of autophagic vacuoles (arrowed) is shown; the DNA content (IOD) is indicated and reveals paradiploidy. Bars=10 μm.

have addressed this conundrum in p53-deficient ETCs induced after genotoxic and spindle damage. Our observations indicate that a proportion of the multinucleated ETCs induced after such damage through mitotic catastrophe do not undergo growth arrest even though they possess such hallmark features of senescence as polyploidy and autophagy. These ETCs, in fact, seem to be able to provide paradiploid mitotic survivors. In particular, the autophagy observed during this process (which is coupled with the chromatin extrusion) is non-senescent in nature, as it is compatible with the mitotic activities of these cells. So, *stricto sensu* senescence is not induced in these cells. Rather, our data suggest that the genomically unstable ETC use this process to rid themselves of excessive and/or irreparable genetic material following a DNA repair and sorting processes that precede polygenomic separation. Furthermore, it appears likely that autophagy is also directly involved in the final stages of depolyploidization when the chromosome bridges linking sub-nuclei are dissolved. These autophagic functions may favour survival of tumour cells after genotoxic damage and are in keeping with reports on the cytoprotective function of autophagy and its contribution to tumour cell survival after chemotherapy (Morselli et al., 2009; Galluzzi et al., 2010).

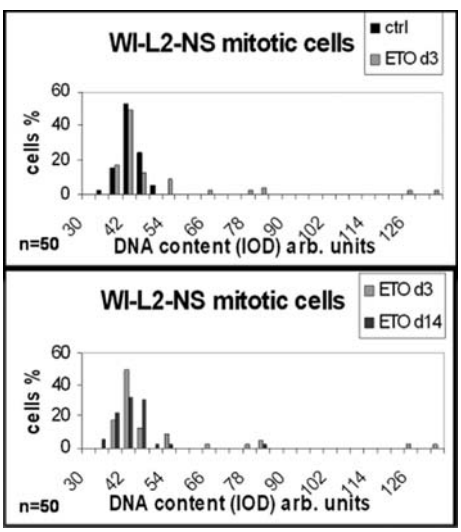

Figure 5 Reduction of aneuploidy in late ETC and their descendents, in the time-course after genotoxic damage

Mitotic DNA content of WI-L2-NS cells at three time points: in non-treated cells, on day 3 after ETO treatment and recovering on day 14 after ETO treatment. WI-L2-NS cells were treated with ETO, and then, the mitotic content of dividing cells was examined 3 and 14 days later and compared with control (Ctl) untreated cells. All day 14 post-ETO mitotic cells bore the remnants of autophagic vacuoles as shown (Figure 4f). Mitotically active paradiploid cells are present in the cell population at this time posttreatment. The graphs show an increase of aneuploidy on day 3 and its decrease on day 14, returning most dividing cells to the paradiploid DNA content. NB: 20% of the mitotic cells on day 14 ETO-treated group displayed deranged disseminated chromosomes and were omitted from these measurements for technical reasons.

Coexistence of this partial chromatin elimination with activation of an embryonal self-renewal programme in ETC is interesting from the ontophylogenic point of view. Partial elimination of chromatin ('chromatin diminution') was first described over 100 years ago by Theodor Boveri in *Ascaris lumbricoides* (Boveri, 1887). It represents one way in which somatic cells in the embryo can undergo differentiation, while the full DNA/chromosome complement remains preserved in the cells of the germline. Diminution has been found in more than 200 families of invertebrates. During this process, whole or selected portions of chromosomes, comprising up to 25–85% of the total DNA, become eliminated from somatic cell precursors (Akifyev et al., 2002; Burt and Trivers, 2006; Kloc and Zagrodzinska, 2008; Gilbert, 2010). In plant hybrids, a

potentially analogous process occurs, whereby a complement of uniparental chromosomes can become assembled, degraded and eliminated (Gernand et al., 2005). In *Tetrahymena pyriformis*, the genomically rearranged chromatin of macronuclei disrupting DNA replication, becomes extruded (reviewed by Raikov, 1995) combining with features of autophagy (Levy and Elliott, 1968) and nucleolysis (Mpoke and Wolf, 1996, 1997). Elimination of mitotic chromosomes through autophagy has been described in human Chang liver cells after a burst of free radicals (Sit et al., 1996). Crucially, in all known cases of chromatin diminution, there is a dependence on cell division (Gilbert, 2010) much as we have seen here in ETCs produced after genomic insult.

As alluded to previously, this particular autophagy may also play a key role in the chromatin-sorting process. DNA sorting in ETC has been suggested previously based upon the presence of micronuclei enriched with Rad 51 (Haaf et al., 1999) and the elimination of amplified DNA sequences by this process (Shimizu et al., 1998). Although not reported as such, these events may be related to the autophagic chromatin extrusion detailed here. Other related important results are experimental observations (Kalejs et al., 2006; Ianzini et al., 2009; Erenpreisa et al., 2009; Vitale et al., 2010) that demonstrate the ability of cancer cells to escape from genotoxin- or spindle-damage-induced mitotic death by activating a depolyploidization programme facilitated by the action of meiosis-specific genes that results in the production of proliferating progenies. In keeping with our findings, Walen (2008, 2010) recently reported cytological observations in pre-senescent cultures of human embryonal fibroblasts, showing both degrading and mitotic subnuclei in the same, albeit rare (1–4%), endopolyploid cells. In addition, chromatin sorting and extrusion might also favour the decrease of aneupolyploidy and genomic stabilization during ETC depolyploidization. Decrease of aneuploidy was described in ETCs of colorectal cancer after nocodazole treatment by Vitale et al. (2010, commented by Erenpreisa and Cragg, 2010) and shown here in lymphoblastoma by DNA image cytometry after ETO irradiation treatments. It may further be aided by the activity of dynamic ELCS moving along the perinuclear space and forming autophagy-inducing loops and pockets enrolling extrachromatin (Erenpreisa et al., 2002); the latest data show that ELCS are engaged in the balanced docking of chromosomes to the nuclear envelope (Olins et al., 2011). Conversely, compromised autophagy was shown to promote DNA damage and aneupolyploidy of tumour cells in response to metabolic stress (Mathew et al., 2009).

Following the chromatin-sorting process in the ETCs, individual subcells become separated and released. Sundaram, Rajaraman and colleagues (Sundaram et al., 2004; Rajaraman et al., 2006) previously interpreted the final budding of subnuclei from disintegrating giant mother cells to be amitotic fragmentation. That interpretation is partially true as autophagic release of daughter nuclei/cells from the chromosome bridges is indeed amitotic. However, our observations (Erenpreisa et al., 2005, 2008, 2009, and here) show that prior to budding, the genetic material has already been segregated in a more regulated manner involving multipolar and bipolar divisions that use meiotic/mitotic machinery (Ianzini et al., 2009; Erenpreisa et al., 2009). Moreover, our observations revealed that chromatin extrusion is compatible with activation of the embryonal self-renewal program and the transfer of its key constituents (OCT4 and NANOG) into the depolyploidized descendents. As we demonstrated through live cell imaging experiments (Ianzini et al., 2009), some of these subcells re-enter normal mitosis and are capable of further propagation ensuring clonogenic survival. However, further studies are needed to clarify the exact role of chromatin extrusion and the sequence of events occurring during ETC divisions.

Importantly, we report here that differentiation of subnuclei is accompanied by a re-synthesis of fresh cytoplasm and the formation of rejuvenated individual subcells, while they still reside within the cytoplasm or detritus of the original ETCs. Presumably, this diversification of subnuclear fate in these ETC can occur by two pathways: (i) by the second FMM, which follows the first and involves only part of the chromatin after its re-sorting between the two events; (ii) by asymmetric bipolar divisions of the subnuclei identifying them as either immortal (extending self-renewal) and mortal through features characteristic of stem cells. Formation of individual cytoplasm territory around the 'stemloid' daughters allows the compartmentalization of the self-renewal/mitotic activators into the individual rejuvenated subcells within the deteriorating mother ETC facilitating the rejection of the excluded subnuclei and old cytoplasm. In this respect, a single ETC at this stage behaves like a developing multicellular system, which may be due to the prolonged activity of embryonal factors. As a result, the new generation is born from, and comes to substitute for, the previous generation of tumour cells. This is the essence of both 'neosis' (Sundaram et al., 2004; Rajaraman et al., 2006) and the 'cancer life cycle' (Erenpreisa and Cragg, 2007, 2010) and reveals how these processes occur cytologically. However, although we have demonstrated this process in several p53 non-functional cell lines, the question remains as to whether primary tumours are capable of developing in this way.

In conclusion, the data presented here further outline the complex cytological processes which occur when ETC undergo depolyploidization and rejuvenation of their descendents through a coupled sorting and autophagic elimination of superfluous genetic material revealing the way (or at least one of the ways) in which these cells are able to return to the clonogenic paradiploidy.

Author contribution

Jekaterina Erenpreisa designed the experiments, carried out microscopical analysis of the material and drafted the manuscript. Kristine Salmina participated in experimental design, carried out immunocytochemistry and analysis of results. Anda Huna carried out DNA image cytometry of WI-L2-NS and analysis of results, and participated in the preparation of the draft manuscript. Elizabeth Kosmacek carried out the experiments and DNA image cytometry on HeLa cells and analysed results. Mark Cragg participated in the design and analysis of experiments and edited the draft manuscript. Fiorenza Ianzini participated in the design of experiments on HeLa cells and edited the draft manuscript. Alim Anisimov carried out mocroscopical analysis of the experiments of HeLa cells, and participated in drafting and editing the manuscript.

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3.6 Macroautophagy-aided elimination of chromatin

Macroautophagy-aided elimination of chromatin

Sorting of waste, sorting of fate?

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How tumor cells process damaged or unwanted DNA is a matter of much interest. Recently, Rello-Varona et al. (*Cell Cycle* 2012; 11:170–76) reported the involvement of macroautophagy (hereon autophagy) in the elimination of micronuclei (MN) from osteosarcoma cells. Prior to that, diminution of whole nuclei from multinucleated *TP53*-mutant tumor cells was described. Here, we discuss these two kinds of chromatin autophagy evoked after genotoxic stress in the context of the various biological processes involved: (1) endopolyploidy and the ploidy cycle; (2) the timing of DNA synthesis; (3) DNA repair; (4) chromatin:nuclear envelope interactions; and (5) cytoplasmic autophagy. We suggest that whereas some MN can be reunited with the main nucleus (through interactions with envelope-limited chromatin sheets) and participate in DNA repair, failure of repair serves as a signal for the chromatin autophagy of MN. In turn, autophagy of whole sub-nuclei in multi-nucleated cells appears to favor de-polyploidization, mitigation of aneuploidy with its adverse effects, thereby promoting the survival fitness of descendents and treatment resistance. Thus, both kinds of chromatin autophagy provide tumor cells with the opportunity to repair DNA, sort and resort chromatin, reduce DNA content, and enhance survival.

Keywords: chromatin autophagy, genotoxic damage, reversible polyploidy, micronuclei, aneuploidy

Abbreviations: MN, micronucleus; NE, nuclear envelope; ELCS, envelope-limited chromatin sheets; NP, nuclear pockets; EM, electron microscopy

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Niels Bohr once said "A great truth is a truth whose opposite is also a great truth." In particular, this should apply to processes which display paradoxically opposing natures. This thought came to mind when reading the article by Rello-Varona et al.[1] concerning the autophagic removal of micronuclei (MN), published recently in *Cell Cycle*. Here, macroautophagy (hereon termed autophagy) was convincingly shown to be involved in the degradation of MN induced in osteosarcoma U2OS cells after cell cycle disruption. The authors suggested that this process may contribute to genome stabilization, however were surprised that the proportion of MN undergoing autophagic processing (~2–5%) was so small. A marginal contribution of autophagy to MN elimination or a rapid turnover of the autophagic MN were suggested as possible causes. Here we discuss several alternative possibilities and more generally consider the various mechanisms involved in the genome stabilization and chromatin autophagy of tumor cells undergoing reversible polyploidy.

Cell-cycle disruptors, such as those used by Rello-Varona et al.,[1] alongside irradiation and other genotoxic insults can induce reversible polyploidy in *TP53* function defective cells which in turn is able to contribute to cell survival. Reversible polyploidy has been observed in various tumor cell lines of mesenchymal and epithelial origin after genotoxic treatment (irradiation, doxorubicin, etoposide, nocodazole, paclitaxel, and cisplatin), (for review and more recent work see refs. 2-6). It is also observed at low frequency in nontreated lymphoma and HeLa cell lines.[7,8] The generality of this process for tumor evolution (also termed 'neosis'[2,9] or the 'cancer life cycle'[10,11]) was suggested previously,

Tetraploidy is associated with genome instability, aneuploidy, and increased mutability serving as a driver of carcinogenesis.[12] Concurrently, aneuploidy has adverse effects on proliferation.[13] Therefore it is expected that the reversible polyploidy caused by anti-cancer drugs should elicit mechanisms which mitigate aneuploidy, reduce mutation load and the associated genotoxic/metabolic stress, thus favoring cell fitness for tumor progression and treatment resistance.[11] As autophagy has been found to reduce DNA damage and stabilize the genome,[14] we therefore propose that chromatin autophagy is involved intimately in this process.

It is well established that *TP53* function deficiency favors endopolyploidy, micronucleation, and resistance in response to genotoxic damage and spindle disrupters. Rello-Varona et al.[1] used U2OS cells which display wild-type *TP53* and *RB1*, but which have lost expression of CDKN2A/p16INK4a. This genotype is equivalent to mutant *TP53*, as the downstream cell cycle suppressors CDKN1A/p21 and CDKN2A are linked with a positive feedback loop.[15] Therefore, as would be expected, all four cell-cycle disruptors employed by the authors displayed enhanced MN, tetraploidy, and higher level polyploidy, followed by a return to diploidy.

MN are routinely observed as a hallmark of genotoxicity and chromosome instability, and result from aberrant mitosis often leading to mitotic catastrophe or mitotic slippage and endopolyploidy. The U2OS cells releasing autophagic MN are seen after mitotic slippage as judged by their nuclear morphology. In these cells autophagic MN are observed alongside clearly non-autophagic MN. Why the autophagic MN were observed so rarely and alongside non-autophagic MN in the same polyploid cell requires further discussion. First, we should consider the link between MN autophagy and DNA repair.

Release of Rad51-enriched MN was previously reported in irradiated HeLa cells by Haaf et al.[16] and for the first time was suggested as being involved in sorting damaged DNA through repair. DNA repair by homologous recombination (HR) is typically enhanced in *TP53*-function deficient polyploid tumor cells and protects them from apoptosis.[17-19] However, although DNA repair by HR is antagonistic to apoptosis, it is still compatible with simultaneous chromatin sorting by autophagic buds from the same cells.[8] In the chromatin buds of endopolyploid cells undergoing intense HR, the DNA recombinases Rad51 and Rad52 are either organized into repair foci or are seen in a disorganized pattern[8,19] (exemplified on **Fig. 1A and B**). In the latter case the DNA in the buds is degraded (low DAPI staining and γH2AFX/γH2AX-positivity) suggestive of failed repair by HR and selective autophagy of that particular chromatin material.[8,20] Support for this association between failed repair and autophagy comes from Robert et al.[21] who recently showed the direct link between the processing of double-strand breaks by repair enzymes and autophagy.

As the autophagic and non-autophagic MN in most cases are seen in close vicinity to the main nuclei of polyploid cells, or are presented as nuclear buds, their relationship with the nuclear envelope (NE) should also be considered. In particular, envelope limited chromatin sheets (ELCS) appear to connect some MN to the main nucleus (exemplified on **Fig. 2A**). ELCS are flat folds of the inner NE attached with ~30 nm heterochromatin fibrils. These folds project into the perinuclear cistern and into the cytoplasm forming nuclear pockets (NP), which then fuse with the nucleus again (for review see refs. 23 and 24; **Fig. 2D**). Therefore, some MN found near the main nucleus may be connected to it by thin ELCS, while some nuclear buds may in fact represent NPs of ELCS with cytoplasmic content. Like MN, ELCS have long been acknowledged as a cytological marker of aneuploidy and associated with poor prognosis in lymphoma and other tumors.[23,24] Also like MN, ELCS appear as a result of aberrant mitosis and/or slippage caused by genotoxic treatment or spindle perturbation.[22]

ELCS enclosing chromatin bound to LBR/Lamin B receptors[24] can apparently transfer chromatin.[23] Although at this point purely conjecture, we therefore propose that some MN which are connected with the main nucleus by ELCS, can be further translocated as an ELCS loop to the NE at a new site to facilitate HR (exemplified in **Fig. 2A and B** and depicted in the scheme in **Fig. 2D**). The translocation of the chromatin by ELCS may occur through nuclear rotation,[25,26] and is apparently enhanced in lymphoma and HeLa cells after DNA damage (unpublished observations). When DNA fails to find homology and bind recombinase, it should signal for selective degradation and budding of the MN for autophagy (as exemplified on **Fig. 2C** and designated in **Fig. 2D**). As a result both non-autophagic and autophagic MN may collaborate in facilitating genome stabilization in endopolyploid tumor cells. This model would explain why most MN are not seen in the process of autophagic degradation—because they are instead engaged in DNA repair and chromatin resorting or are otherwise disconnected from the nucleus.

As noted by Rello-Varona et al.[1] the MN autophagy caused by cell-cycle disrupters occurs on a background of ongoing cytoplasmic autophagy. In addition, activated mitochondria and cytoplasmic autophagy are frequently observed in the cytoplasmic pockets of ELCS which are internally digested in endopolyploid lymphoma cells after genotoxic treatment[22] as shown in **Figure 2B** and designated in **Figure 2D**. Therefore, cytoplasmic autophagy may partner with chromatin autophagy in the maintenance of genome stability by at least two mechanisms: (1) by supporting and extending the viability of the cell as it undergoes protracted DNA repair; and (2) more directly, by providing the energy needed for nuclear rotation by microtubules at the site of the ELCS pockets.

Somewhat similar autophagic digestion of chromatin by nuclear envelope buds and formation of perinuclear autophagic vacuoles has previously been described in several disease envelopathies and corresponding in vitro models.[27] It follows then that these phenomena are induced by genome instability on one hand, and nuclear envelope instability, on the other, highlighting the role of the chromatin-nuclear envelope relationship in maintaining genome integrity and order. Another phenomenon of nuclear autophagy related to the NE has been described in yeast; named piecemeal microautophagy of the

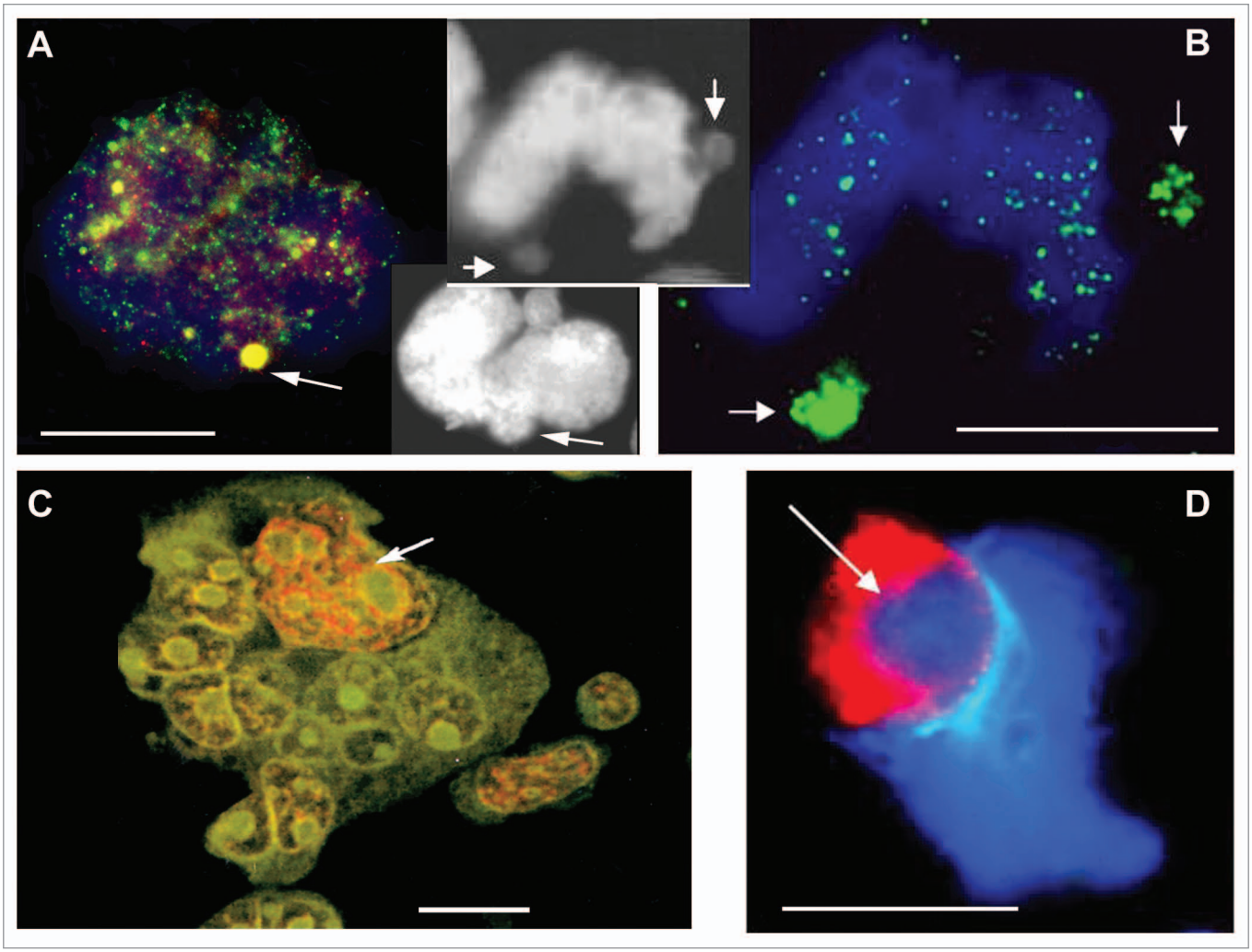

Figure 1. (**A and B**) Characterization of the association between MN, homologous recombination repair and DNA integrity in *TP53* mutant lymphoma cells after γ-irradiation insult. (**A**) A polyploid nucleus undergoing intensive DNA repair by HR (testified by multiple repair foci positive for Rad51 and γH2AFX/γH2AX). A MN (arrow) is being released which involves a large repair focus. The corresponding DAPI gray-scale image (insert below) shows no loss of DNA content in this MN. (**B**) A polyploid lymphoma cell undergoing both DNA repair by HR (as testified by the presence of multiple Rad52-positive foci) and simultaneous release of two MN (arrows) containing large aggregates of Rad52 and degrading DNA, as seen by the reduced DAPI content (shown in the insert to the left); (**C and D**) release of whole sub-nuclei from polyploid lymphoma cells showing (**C**) selective chromatin degradation of sub-nuclei seen using the acridine orange in situ denaturation test [where red fluorescence indicates degraded DNA (arrowed) and green fluorescence indicates intact DNA] and on (**D**) sequestration of large amounts of DNA (DAPI stained blue) in a perinuclear vacuole accumulating CTSB/Cathepsin B, indicative of autophagy (red, arrowed). Bars: 20 μm. Figures are republished with new annotation: (**A**) from reference 19 and (**C**) from reference 20, with permission of Elsevier; (**B and D**) were originally published in reference 8, copyright holder Portland Press.

nucleus (PMN).[28] Here tiny pieces of the nucleus are invaginated in the apposed perinuclear vacuole with subsequent scission and digestion of the enclosed material; the terminal stage of the process requires the core macroautophagy genes.[29] During this process, excess pre-ribosomal material from the nucleolus is mostly eliminated, alongside chromatin. Amplified rDNA may also be removed and if so, PMN would serve to contribute to the stabilization of the genome that we propose occurs in mammalian cells.

As detailed earlier, chromatin autophagy is related not only to MN. Selective degradation, autophagic digestion and expulsion of whole sub-nuclei also occurs in endopolyploid tumor cells[8,20] (as shown on **Fig. 1C** and **Fig. 1D**). This activity was seen in several lymphoma and also HeLa cells after different treatments (irradiation, paclitaxel, nocodazole, etoposide) and coincides with de-polyploidization by a-cytotomic multi-polar mitoses. In contrast to autophagy of the MN during the polyploidization phase where DNA synthesis can be concurrent,[20,22] whole sub-nuclei prepared for autophagy selectively halt DNA synthesis and become degraded and extruded.[8,20] Subsequently, a proportion of the retained sub-nuclei not only maintain DNA synthesis but also accumulate the self-renewal factors POU5F1/Oct4 and Nanog, sequester their own cytoplasm and then dissipate as mitotic descendents.[8,20,30] These features associated with the elimination of whole sub-nuclei, namely cessation of DNA synthesis, TUNEL-positivity,

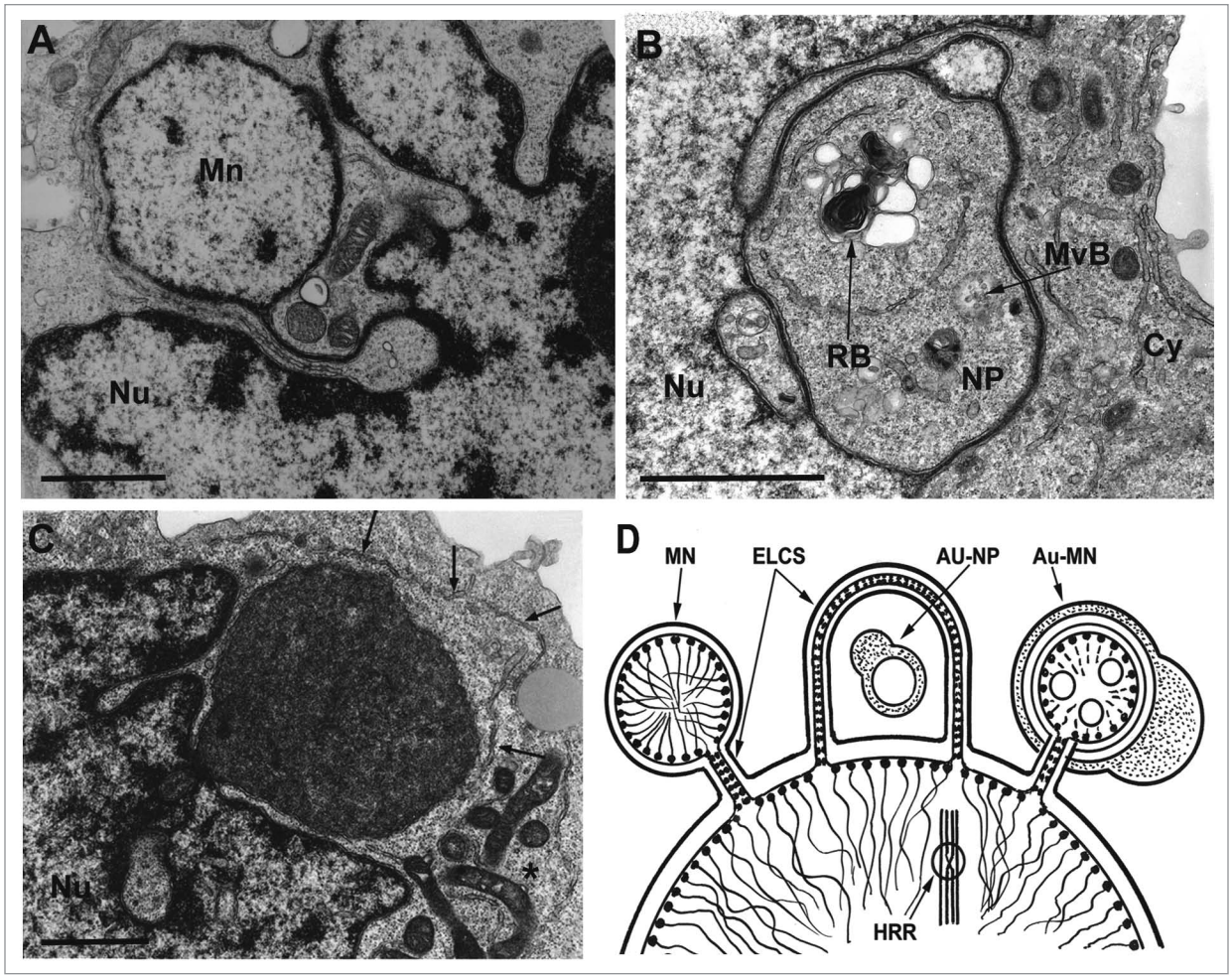

Figure 2. The relationship between MN, NE, ELCS, DNA repair and chromatin autophagy in endopolyploid cells undergoing genotoxic stress. (**A**) A MN with normal chromatin structure is linked to the nucleus (NU) by ELCS. (**B**) In the nuclear pocket (NP) the dark organelles with convoluted membranes represent the residual body (RB), which usually results from autophagic lysosomal activity. The sequestration by a double membrane and the presence of a multivesicular body (MvB) nearby are also indicative of cytoplasmic autophagy within the NP; Nu, nucleus; Cy, cytoplasm. (**C**) Extrusion of a large membrane enclosed MN containing degraded chromatin via the nuclear pore. Note the sequestration of the cytoplasmic territory around it by a double membrane (arrows) and assembly of activated mitochondria nearby (asterisk). Bars: 1 μm. Figures are republished with new annotation: (**A and B**) from reference 22, with permission of Springer and (**C**) from reference 20, with permission of Elsevier. (**D**) A schematic showing a cross-section of a nucleus undergoing micronucleation and chromatin autophagy. Interphase chromosomes are joined to the NE by heterochromatin rows attached to the LBR of the inner nuclear membrane, which form the chromatin band of the ELCS. The left micronucleus (MN) is connected to the nucleus by ELCS and is not autophagic; it can be reunited with the nuclear DNA or alternatively form an ELCS nuclear pocket bridging to the nucleus at another site. The process may favor the search for homology for recombination repair of double-strand breaks in the foci (HRR). The cytoplasmic content of the nuclear pocket often undergoes autophagy (Au-NP) (designated by sequestration of the double membrane fusing with a lysosome). If DNA repair has failed (and DNA remains fragmented), a signal for budding of autophagic MN (Au-MN) may be obtained from unbound repair factors (illustrated as free rings OOO) for execution of selective autophagy (designated by sequestration of the double membrane fusing with a lysosome).

involvement of lysosomal enzymes, and active expulsion are somewhat in common with those described for the elimination of the vegetative macronucleus in unicellular *Tetrahymena*,[31] perhaps indicating an evolutionary origin for this process in tumors. Presumably such autophagic processes also provide the nutrients and energy for the surviving secondary subcells thus favoring the ultimate escape from genotoxic damage. A similar nutritive role was suggested for the autophagic elimination of whole nuclei in syncytial filamentous fungi.[32]

In conclusion, the role of autophagy in the maintenance of genome stability is well established by genetic and other mechanistic studies,[14,21,28] however, the understanding of the specific contribution made by chromatin autophagy is only just beginning. Here, we underline the significance of polyploidy, and reversible endopolyploidy in particular, for chromatin autophagy, highlight the regulatory role of DNA repair and regulation by the nuclear envelope and discuss the differing kinds of chromatin autophagy, by MN and release of whole sub-nuclei in this process. Exactly how all of these various facets are coordinated to regulate genome stability

and ultimately tumor cell fate will clearly require further research.

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3.6.1. DNA extrusion in ETCs after different DNA damaging treatments

Selective extrusion and further autophagic degradation of DNA occurs in endopolyploid tumour cells (ETCs) after different treatments (irradiation, paclitaxel, nocodazole, etoposide) and is positive for autophagic marker LC3B as found in PA1 cells (Figure 9).

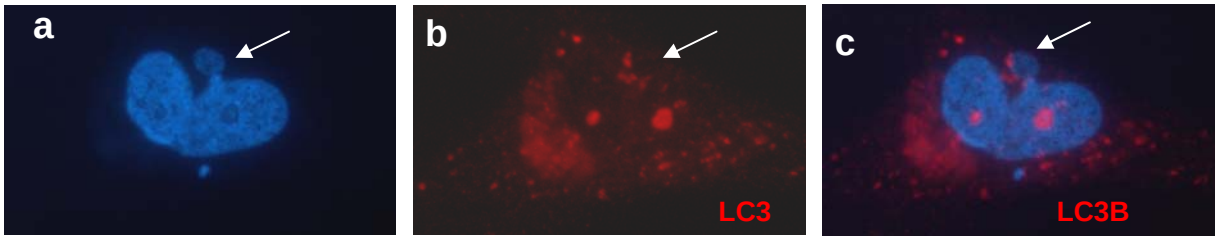

Figure 9. Chromatin (DAPI-stained) degrading in autophagic vacuoles labelled by LC3B in polyploid tumour cells after etoposide treatment.

4. Discussion

The previous study in our laboratory showed that recovery of TP53 mutant tumour cells after genotoxic damage is associated with induced polyploidy and its reversal (Erenpreisa *et al.,* 2000; 2005, 2008). This process of induced polyploidy was suggested to be cyclic and the hypothesis of the cancer cell life cycle as an ilicit transition from the mitotic cycle to cycling polyploidy was postulated (Erenpreisa and Cragg 2007). To exert something similar to a life cycle, cancer cells should activate the genes responsible for the main features of meiosis and early embryogenesis. The previous observations showed that irradiation up-regulated expression of meiosis-specific genes such as REC8, MOS, SPO11, Stag3 and SCYP1, 2, 3 (Kalejs *et al.,* 2006; Ianzini *et al.,* 2009; Vitale *et al.,* 2010) at the background of TP53 deficiency.

Here we showed for the first time using human lymphoma and cervical carcinoma cell lines that polyploidy induced by DNA damage in somatic tumour cells, indeed, induces the embryonal-type stemness at the TP53-function deficient background and also documented cytologically the main features of mitotic-to-meiotic transition in them, both leading to escape by cycling self-renewal. These findings pour more light on the well known fact on the significance of TP53 function deletion and its link to polyploidy for tumour aggression and resistance to treatments and would explain the data accumulated on the expression of stemness key cassette (OCT4, SOX2 and NANOG) in various tumours (Ben-Porath *et al.,* 2008; Saigusa *et al.,* 2009; Ge *et al.,* 2010; Xiang *et al.,* 2012) and on cancer testes-associated antigens (CTA) and clearly meiotic genes, such as MOS, REC8, SPO11 in lung cancer and melanoma (Fratta *et al.,* 2011, Rosa *et al.,* 2012.) associated with poor clinical prognosis.

More particularly, in respect of meiotic cohesin REC8, we observed not only its expected localisation between centromeres of sister chromatids but, for the first time, also found the unusual for meiosis location of REC8 in the centrosome of interphase ECTs and at the spinde poles of giant ETCs undergoing bi-or multi-polar divisions. While in common with meiosis, the binding of REC8 to centromeres and localization between a pair of centromeres was found, localization of REC8 along chromosome arm in prophase was not found. So, the found intracellular distribution of REC8 has the most critical meiotic features necessary for cosegregation of sister chromatid for reduction division combined with the features aberrant for mammalian meiosis. However, in yeast meiosis, the stabiliser of REC8 cohesion to centromeres – Shugoshin, was recently shown to associate also with centrosomes (Gutiérrez-Caballero *et al.,* 2012). So, association of REC8 to a centrosome (which serves an integrative checkpoint to DNA damage response) in lymphoma cells may represent an evolutionary pattern of meiosis regulation, as the latter originated from the DNA damage response (Bernstein *et al.,* 1987).

Irradiation of lymphoma cells caused increased expression, post- translational modification and stabilisation of REC8, indicated by the appearance or stabilisation of a 90 kDa protein form in Western blots. In line, the post-translationally modified products were also described in mouse meiosis (Eijpe *et al.,* 2003).

Secondly, the DNA breaks, detected in polyploid cells on days 4 to 6 after irradiation, often colocalised with the foci of meiosis-specific recombinase DMC1, indicating for attempted DNA repair by homologous recombination (HR). Interestingly, Ianzini *et al.* (2009) reported the transcriptional upregulation of another meiosis-specific gene- SPO11, which is classically associated with meiosis initiation and physiological DSB formation, and also DMC1 foci formation in HeLa and MDA-MB435 cell lines 4 days after irradiation, as well as accumulation of DMC1 in tissue specimens from human cervical cancer. It is possible that a feature of DNA repair could be used by ETCs to bring homologous chromosome together, at

least in some ETC. Co-segregation of sisters occuring in ETC is likewise the first division in meiosis. It should be noted that cosegragation of sister chromatids is a very permanent feature in bi-polar divisions of tetraploid cells induced by senescence or DNA damage as also described by other authors (Walen, 2008; Davoli *et al.,* 2010).

It seems likely that a REC8 containing cohesin complex involving SGOL1 could mediate sister centromere cohesion in ETCs that resume cell divisions >5 days after irradiation. SGOL2 was also expressed in the lymphoma cells both before and post IR, further supporting the observation that ETC as such express the capacity for meiotic-like ploidy reduction. Such a mode of reductional segregation akin to that seen in meiosis I may contribute to the first step in the ordered genome reduction in the IR-induced ETCs thereby creating surviving tumour cells that escape radiation therapy.

It appears that in ETCs genome segregation of the chromosomes by meiosis- like mechanism involving cohesion of sister chromatids by REC8 (presuming synapsis of homologous chromosomes, which is more effective in DSB repair) can occur albeit not seen in each dividing cell, while division of tetraploids with cohesed sisters as such is seen regularly. It is possible that meiosis-like behaviour induced in tumour cells by irradiation bears some features of its evolutionary origin from ploidy cycles, requiring cohesion of sisters, however optional for recombination between homologs (Kondrashov 1994) or that only those rare cells which procede true meiosis can further produce clonogenic descendents. This would explain low recovery rate by reversible polyploidy 10^{-4} – 10^{-6} cells (Puig *et al.,* 2008; Lee *et al.,* 2009). Mechanistic experiments to elucidate this point are further needed.

Importantly, we report here for the first time, that induced polyploidisation is associated with up-regulating OCT4, presuming induction of nuclear reprogramming. It was seen most spectacularly in lymphomas with constitutively activated c-MYC, however, at a smaller scale was also observed in HeLa cells.

As we found that not only OCT4A, but also different OCT4 isoforms and pseudogenes being activated, they may be involved in the response of lymphoma cell lines after irradiation. The most important finding was upregulation of NANOG - a down-stream target of the key pluripotency and self-renewal genes OCT4 and SOX2, which was needed mutant TP53 dependent. Up-regulation of the NANOG promoter is known to be associated with self-renewal symmetrical mitotic divisions (Silva *et al.,* 2009). Therefore, our finding of NANOG foci symmetrically distributed within the cytoplasm of dividing daughter cells recovering after DNA damage may be interpreted as a hallmark of such divisions.

We also found for the first time that nuclear PML bodies structurally integrate the key embryonal transcription factors SOX2, NANOG nucleated by OCT4 and likely favour growth of the intranuclear network of these transcriptional factors. This observation is in accordance with recent data provided Chuang *et al.* (2011), that functional role for PML is to maintain a specific open chromatin conformation of the OCT4 promoter region for its constant expression in stem cells (Zhou and Bao 2013).

The role of polyploidisation in activation of these transcription factors after genotoxic stress can be associated with increased gene dosage effect, because for starting the positive feed-back circuit of transcription activation reaching a threshold level of ESC factors is required. However, although the transcription is enhanced in general, the balance between genes also becomes changed by polyploidy as shown by transcriptome studies of polyploidy hepatocytes and kardiomyocytes, f.ex. by favouring metabolic shift for enhanced transcription of c-myc and many other proto-oncogenes and decrease of aerobic respiration (Anatskaya *et al.,* 2013). The latter is an important cancer hallmark (Hanahan and Weinberg 2011).

Althogether our observations indicate that self-renewal of lymphoma cell lines might occur by reactivation of a transcriptional programme similar to that engaged during oocyte maturation followed by early embryogenesis (recapitulation of phylogenetic trait akin to

parthenogenesis) through activation of largely germ-line specific genes and their functional pseudogenes. Recent research on hepatocellular carcinoma cell lines and also using breast cancer cell lines and patient samples showed, that stemness can be induced in non cancer stem cells by exposure to ionizing radiation or chemotherapy, thus confirming our data (Lagadec *et al.,* 2012, Ghisolfi *et al.,* 2012 Abubaker *et al.* 2013). As well, there is a growing evidence concerning the role of polyploid cells in tumourogenesis. Lagadec *et al.* (2012) showed that reprogramming of breast cancer cells to the pluripotency state by ionising irradiation occurs in the induced polyploid subpopulation proportionally to irradiation dosage. Zhang *et al.* (2013) revealed that in response to hypoxia stress induced polyploid cell can form cancer (stem-like) spheroids in vitro and generate tumors in immunodeficient mice by de-polyploidisation of even a single giant cell. The high tumorigenicity of single giant cells was also found by Weihua in osteosarcoma (Weihua *et al.,* 2011). All this data confirm and generalise our findings.

The discrepancy between OCT4 up-regulation and NANOG down-regulation, found by us in wtTP53 TK6 cells is possibly associated with two different functions of OCT4: (1) it is a TP53 dependent participant of the legitimate DNA damage response in ESC cells as shown in our recent study (Jackson *et al.,* 2013) and by Bartova *et al.* (2012) who found reqruitment of OCT4 to the DSB, and (2) it serves as a driver of illicit reprogramming of somatic tumour cells to ESC state, when down-regulation of the Nanog promoter by p53 is cancelled by mutation of TP53. It is well known that deletion of TP53 function is the most crucial step to cancer progression (Carson and Lois 1995). The cells like TK6 are very radiosensitive and cannot escape DNA damage through reversible polyploidy coupled to reprogramming. Therefore restoring function of TP53 is a promissive way of cancer treatment (Chen *et al.,* 2010).

It is interesting that not only OCT4B but also meiotic proteins MOS (Vitale *et al.,* 2010 and own unpublished observations) and REC8 were found in centrosomes of resting and dividing giant tumour cells. Centrosome is an integrator of the DNA damage response (Golan *et al.,* 2011). Therefore preliminary, this data suggests that OCT4B together with meiotic factors may participate in coordination of mitotic to meiotic transition through DNA damage checkpoint mimicking the oocyte-maturation-like state and induction of the embryonal-type pluripotency in tumour cells.

Although the proportion of the multinucleated ETCs induced after genotoxic damage possess such hallmark features of senescence as polyploidy and autophagy, they do not undergo terminal growth arrest. Moreover, autophagy and chromatin extrusion are compatible with division activity of depolyploidising cells and accumulation of its key transcription factors - OCT4 and NANOG, into the depolyploidized descendents, which acquire motility and leave old mother cells. Interestingly and in accord, Rausch *et al.* (2012) reported that autophagic markers co-localize with CSC markers in tumour tissues, which were derived from pancreatic cancer patients. Also it is found that autophagy is mehanistically linked to the maintenance of breast cancer cell population expressing stemness - high levels of CD44 and low levels of CD24 (Cufi *et al.,* 2011).

In particular, autophagy may play a key role in the chromatin-sorting process. DNA sorting in ETC has been suggested previously based upon the presence of micronuclei enriched with Rad51 (Haaf *et al.,* 1999). We suggest that genomically unstable ETC use autophagy to get rid of excessive and/or irreparable genetic material following the DNA repair and sorting processes. Chromatin sorting and extrusion might also favour the decrease of aneupolyploidy and genomic stabilization during ETC depolyploidization. Decrease of aneuploidy was found in mitotic cells released from ETC after etoposide treatment and similar results were described in ETCs of colorectal cancer after nocodazole treatment by

Vitale *et al.* 2010. This process may counterbalance excessive aneuploidy preventing cell divisions, thus both driving tumour progression.

In main features, our findings are consonant to the data obtained by Sundaram *et al.* (2004) by life-imaging, where the budding of subnuclei from disintegrating and senescing giant mother cells were observed. The new features found in our research is that prior to budding, the genetic material has already been segregated by multipolar karyotomy (Erenpreisa *et al.,* 2005, 2008, 2011) and that the DNA repair process preceded sorting of the nuclei by autophagy leaving behind rejuvenated nuclei with enhanced stemness properties and capability to form individual cells. Similar to our data were recently provided by Zhang *et al.* (2013), which used sorting of polyploid cells of the ovarian carcinoma in hypoxic conditions and observed releasing of small cells with epithelial to mesenchyme transition (EMT) phenotype from giant mother cell.

Importantly, we report here for the first time, that sequestration in autophagosomes of the diminuted sub-nuclei is accompanied by re-synthesis of ergastoplasm around the rest sub-nuclei and the removal of the external cytoplasm of the polyploid mother cell. Perhaps, it serves to reduce the immunoreactivity of the rejuvenated descendents that are released (Erenpreisa and Cragg 2013).

Formation of new „stemloid" daughter cell within the old ETC is in accordance with suggestion of Blagosklonny (2006) on reprogramming of somatic tumour cells and with both 'neosis' (Sundaram *et al.,* 2004; Rajaraman *et al.,* 2006) and the cancer cell 'life cycle' hypotheses (Erenpreisa and Cragg, 2007, 2010). In fact, this is a new twist of the centuries old embryological theory of cancer (Erenpreiss 1993) complemented with reversible polyploidy (Erenpreisa and Cragg, 2013).

4. Conclusions

1. Meiotic cohesin REC8 is ectopically expressed in mt TP53 irradiation-induced lymphoma ETCs where its localisation to kinetochores and interphase centrosomes was found. In meta- and anaphase ETCs, REC8 additionally localises to the spindle poles, suggesting a role of REC8 in the coordination of the endopolyploid centrosomal and chromosomal cycle.
2. ETCs with the compromised TP53 function ectopically express main features of meiotic divisions (sister chromosome cohesion and omitting one DNA replication cycle), which can contribute in recombination and reduction of chromosomes.
3. Up-regulation of the pluripotency and self-renewal transcription factors OCT4, SOX2, NANOG (cell reprogramming to the ESC-like state) is induced by irradiation in polyploid TP53 mutant lymphoma cells.
4. Self-renewal response to DNA damage, and also polyploidy, is not realised or is very abortive in wt TK6 lymphoma cell line.
5. PML bodies are involved in formation and stabilization of the genotoxic stress induced ESC core transcriptional network
6. Centrosomes accumulating OCT4B, REC8 and MOS possibly integrate the DNA damage-induced meiosis-like cell cycle arrest and divisions with the induction of pluripotency.
7. Autophagy takes part in chromatin sorting, diminution, release of chromosome bridges between sub-nuclei of ETC and thus is involved in upregulation of stemness and self renewal factors, thus pointing to potential role of autophagy in tumour cell survival after genotoxic stress.

5. Main theses for defence

1. DNA damage causes activation of the meiotic-like program, in particular activation of the functional meiotic cohesin Rec8 and omission of DNA synthesis in divisions of somatic TP53 mutant endopolyploid tumour cells.

2. DNA damage causes (activation of self-renewal pathway) reprogramming to the ESC-like state in association with induced polyploidy and favour survival of de-polyploidised descendents from somatic TP53 mutant tumour cells.

3. Germline induction (meiotic and pluripotency) in somatic polyploid tumour cells is prohibited by functional tumour suppressor TP53.

4. Chromatin selective destruction and autophagy are part of survival strategy of the damage-induced polyploid tumour cells concordant with up-regulation of self-renewal in de-polyploidised descendents of the same endopolyploid cells.

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