

## Research Article

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## Inhibition of Apoptosis in *ALL-1* Leukemic Cell Lines: Allowance of Replication, Constant Repair Replication, Defect DNA Damage Control

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### Abstract

The central biochemical events in tumor induction are not completely understood. In a novel cell biochemical approach some questions are answered here. A proliferative mutation allows replication shortcuts to the S-phase in the cell cycle. The repair system is involved in tumor formation, and many tumor cells cannot be brought to apoptosis induction. This study illuminates the biochemical pathways of these features. Mutational translocations within the gene *ALL-1* might cause human acute leukemia. In those leukemic cell lines, replication starts immediately after mitosis. Mutations thus, allow replication without control, defining *ALL-1* as the proliferative gene. Cells metabolize their DNA continuously. The repair system is constantly active. Cells are blind to DNA damage by methylating agents and then replicate and repair their DNA. Induction of apoptosis fails. Thus, chemotherapy resistance is intrinsic. Tumor induction occurs by a mutation that allows replication and turns on the repair system.

**Keywords:** *ALL-1*; Tumor formation; Repair replication in tumor cells; Proliferative genes; DNA repair; Hydroxyurea; DNA damage; MMS; Cisplatin; Bleomycin; Cytosine- $\beta$ D-arabinofuranoside; Apoptosis

### Introduction

Tumor formation lies in gene defects that alter the normal program of cell proliferation. Presumably similar gene defects are responsible for tumor initiation in animals and humans. A genetic system to achieve insight into interactive pathways within a manageable time frame is represented by *Drosophila*. Tumor-forming flies were studied by genetic analysis for twelve years. From several identified mutant genes that contribute to tumor formation, those defects have been found that are responsible for loss of control over the cell cycle: the proliferative genes [1]. These gene defects allow cell division in cell-cycle-competent cells and produce aberrantly over replicated giant chromosomes in other cells. Allowing replication shortcuts the cell cycle control, i.e. shortcut from G1 to S-phase checkpoints. In addition, proliferative mutations in *Drosophila* result in fluffy chromosomes, meaning the chromatin is not packed as usual. In certain regions, specific for the gene defect, chromosomes are not somatically paired, thus a local structural component of the chromatin is affected. Chromosome pairing is involved in two known cellular processes: DNA recombination and DNA repair. Proliferative mutations lead to local inappropriate over-replication of the genome i.e. they induce genome instability [2]. One single proliferative mutation is sufficient to induce tumor formation, somatic pairing defect and genome instability.

About ten different proliferative genes can be identified on one chromosome. They do not carry repetitive DNA, but interfere genetically. The expression profile shows many poly-A transcripts in one direction and one poly-A transcript in the opposite direction. None of this poly-A transcripts is clonable. The related genomic DNA can be cloned, but bacteria carrying this DNA show poor and abnormal growth. Electron microscopic evaluation revealed that their genome is not ordered as one or two light spots in one bacterium, but spread in several smaller spots throughout the bacterium. Bacteria are hyperlong, thus cell division is affected, a phenotype similar to *recA* mutants. All together it is expected, that proliferative genes are involved with origins of replication (data not published). Neither oncogenes, tumor suppressor genes, nor switch genes alone or in various combinations thereof are able to break the cell cycle [3,4].

All thus far identified oncogenes, tumor suppressor genes, and

switch genes in *Drosophila* have homologue genes in the human genome. The equivalent human genes were identified to be involved in tumor formation. The *Drosophila* gene *trithorax* interferes with the switch gene *Antennapedia* in *Drosophila* and is related to the human *ALL-1*(*MLL1/HRX*) [5]. *ALL-1* gene mutations participate in acute leukemia in 5% to 10% of children and adults. Like other genes, *ALL-1* was discovered by the association with chromosome rearrangements, involving its location in chromosome band 11q23 [6]. Molecular analysis has revealed a mutation called "self fusion". Those mutations that lack duplicated zinc finger motifs are associated with trisomy 11 [7]. Thus, mutation of the gene might be involved in allowing replication of chromosome 11.

*ALL-1* encodes for a protein of 431 kDa consisting of 3972 amino acids [8,9]. Protein motifs provide clues to possible *ALL-1* functions. An AT-hook motif is present, involved in protein binding to the minor groove of DNA and binding to oriG1ns [10,11]. *ALL-1* shows methyltransferase activity. AT hooks and the methyltransferase are duplicated in self fusion mutations. Both motifs are present in all fusion proteins causing acute leukemia. Fusion of both motifs to bacterial beta-galactosidase leads to a tumorigenic protein [12]. These points towards their importance in tumor formation [13].

DNA methyltransferases in mammals imprint on chromatin structure. They may participate in the repair of certain unusual structures by serving as a nucleation point, binding to DNA defects. They may participate in chromosome remodelling by stabilizing RNA-DNA hybrids or RNA-RNA secondary structures. They may there be involved in replication initiation [14]. In *Drosophila*, DNA methylation to imprint chromatin structure is absent, whereas DNA methyltransferase activity to initiate DNA repair is functional [15,16].

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Involvement of the repair system in tumor formation has been anticipated because defects in DNA-repair genes like BRCA predispose to cancer [17,18]. BRCA1 associates with Rad51, a protein known to be involved in homologous pairing of chromosomes [19,20].

Essentially three different DNA repair pathways are induced by DNA damage. Recombination repair is induced by double strand breaks. Gamma irradiation or radiomimetic drugs like bleomycin lead to DNA scission and induce this pathway, which strongly depends on somatic pairing of homologous chromosomes. A second pathway is inducible by UV-light, leading to thymidine dimers, which are mostly eliminated by nucleotide excision repair. Alkylating agents that crosslink DNA, can induce mismatch repair, which requires specific enzymes to excise defect bases. Both latter mechanisms, nucleotide excision and mismatch repair, do not ultimately depend on homologous replication of chromosomes [21,22]. Methylating agents like methanemethylsulfonate (MMS) induce both latter pathways; additional synergy to recombination repair is anticipated [23].

The central biochemical events in tumor induction involve replication and repair, origins and chromatin structure, somatic pairing and genome stability. In a novel approach, the correlation of the initial biochemical events is illuminated. As found in *Drosophila*, a proliferative mutation allows replication shortcuts to the S-phase. Can this also be the cause of human tumor formation? How is the repair system involved, and how is the switch to apoptosis defective? This study illuminates the biochemical pathways of these features. Measurements of the biochemistry within tumor cells concerning replication, repair, and apoptosis defects are presented.

## Materials and Methods

### Cells

Nalm6 (human lymphoblastic leukemia), U937 (human histiocytic lymphoma), and K562 (myeloid leukemia) do not carry *ALL-1* translocations. Leukemic cell lines (*ALL-1* tumor cells) B1B, SEM, MV4; 11 and RS4; 11 carry the translocation of *ALL-1* to AF4, i.e. t(4;11)(q21;q23). ML2 carries the translocation to AF6 t(6;11)(p22;q23). THP1 and MonoMac6 carry the translocation to AF9 t(9;11)(p22;q23). Cells were obtained from Eli Canaani and maintained in RPMI-1640 (Sigma) containing 2% antibiotic mixture and 10% fetal calf serum (Sigma) at 37°C under 5% CO<sub>2</sub>.

### Synchronization of cells, mitotic count

Demecolcine (Sigma) is stored at -20°C as 1 mg/ml stock solution for less than a month. It inhibits the formation of the cellular spindle and arrests cells in metaphase of mitosis. Cells are starved for 40 hours in medium without fetal calf serum, then boosted for 8 hours with 10% fetal calf serum, and incubated with 1 µg/ml demecolcine for 16.5 hours. Cells are then centrifuged for 5 minutes at 1000 × g and washed once with culture medium and released in fresh medium. After 4-5 hours mitosis takes place for about one hour, and then cells enter the G1 phase. For mitotic count, 1 ml of cells are harvested for 5 minutes at 3000 × g and resuspended in 100 µl of 0.7% NaCl. After incubation for 10 minutes at 22°C, 5 µl of fresh methanol:acetic acid 3:1 is added prior to centrifugation. The cells are spread and stained with 0.2% orceine in 45% acetic acid.

### DNA structure analysis

1 ml cells are centrifuged at 13,000 × g and washed once in phosphate-buffered saline, pH 7.5 (PBS). Cells are resuspended in 400

µl STE (100 mM NaCl, 20 mM Tris-HCl pH 7.4, 10 mM EDTA pH 8.0, 0.5% SDS, 0.2 mg/ml Proteinase K) and incubated for 15 minutes at 37°C. 120 µl 5M NaCl is added, and the mixture is centrifuged for 5 minutes at 13,000 × g. 400 µl of the supernatant is transferred to 1 ml of -20°C ethanol. DNA is pelleted for 5 minutes at 13,000 × g; the pellet is washed once with 70% ethanol, dried, and then dissolved in water. DNA is incubated at 96°C and chilled on ice to obtain single strands prior to agarose gel electrophoresis.

### Kinetics of uptake of nucleotides into cells and incorporation into nucleic acids (uptake rate, *in vivo* replication rate, transcription rate, pulse and chase)

Prior to the experiment, cells are incubated for 24 hours in fresh medium to ensure exponential growth. Prior to each experiment cells are diluted 1:3 in fresh medium. 1 µCi <sup>3</sup>H Thymidin (3HT)(5 Ci/mmol, 1 µCi=200 pm; ICN) is added per 50,000 cells in 10 ml. This quantity allows the measurement of replication rates within a linear range for about 40 hours without deprivation of thymidine. Incubation times are from 3 hours to 30 hours for each experiment. Usually every 30 minutes, samples are taken.

To measure the transcription rate, <sup>3</sup>H Uridine (3HU) is used the same way (36 Ci/mmol, 1 µCi=28 pm; ICN). Two independent 0.4 ml samples are taken at each time. Each experiment is usually carried out twice. UPTAKE RATE: Cells are collected by centrifugation for 5 minutes at 3000 × g. The supernatant is removed, and 100 µl of it is used for liquid scintillation counting (LSC). Cells are washed in PBS, lysed in STE and used for LCS.

### Replication, repair and transcription rates

Radioactivity incorporated to the nucleic acids is determined as follows: Cells are harvested by centrifugation for 5 minutes at 3000 × g and resuspended in 100 µl STE. After incubation at 37°C for 30 minutes, 300 µl (500 mM Na acetate pH 5.0, 10 mM MgCl<sub>2</sub>, 86% ethanol) is added and samples are collected at -20°C. After incubation overnight nucleic acids are harvested at 15,000 × g for 15 minutes and washed once with 70% ethanol. The pellet is dried and dissolved in 100 µl H<sub>2</sub>O. 800 µl scintillation fluids (Ultima Gold, Packard) are added, prior to LSC. All samples are measured at least twice for at least one minute to minimize counting errors. As result for one point, the arithmetic average of two (times counting) times two (independent samples) is taken. Nucleic acids are quantified by dissolving the nucleic acid pellet in 1 ml water, and absorbance at 230, 260, and 280 nm is determined. Pre-experiments showed that 80-90% of the thymidine or uridine transported into the cell, is immediately bound to nucleic acids.

Drugs and chemicals used for damage induction and repair and replication analyses are summarized in table 1. Pulse and Chase: For determination of excision rates (instability of 3HT in nucleic acid), cells are incubated for 1 hour with 3HT (pulse). At time 0, and 1 hour, samples are taken and analyzed with LSC. Cells are washed once with medium and incubated for 1 hour in fresh medium without 3HT (chase). At beginning and end of the chase, samples are taken and analyzed with LSC. Measurement of the overall DNA content at the end of pulse and beginning of chase allows normalization of the data at this point. Release rate during the chase is related to incorporation rate during pulse.

### Determination of the lethal dose of the drugs and chemicals

Cells are incubated with different concentrations of MMS,

Name/Abbreviation	Source	Effect
<b>Damage Induction</b>		
Bleomycin sulphate (Bleo)	ICN	cause DNA scission and induce recombination repair. a)
Diamindichloridoplatin/Cisplatin	ICN	inhibit replication through crosslinking of two guanosine b)
Methylmethanesulfonate/MMS	Sigma	Methylation of DNA c)
<b>Distinguish Replication from Repair Synthesis</b>		
Hydroxiurea (HU)	Sigma	inhibit 95% of replication, repair is unaffected d)
Cytosine-βD-arabinofuranoside (AraC)	Sigma	inhibit replication e)
Aphidicoline (Aph)	Sigma	inhibit 50% of Bleomycin induced repair synthesis f)
2'3' deoxythymidine (ddT)	ICN	inhibit 80% of Bleomycin induced repair synthesis g)

a) Bleo was dissolved to 10 U/ml and stored at -20°C. End concentration is 0,1 U/ml. This induces visible DNA damage, as revealed by DNA structure analysis after incubation of 1 hour.

b) Cisplatin was dissolved in DMSO as 50 mM solution prior to use. End concentration as indicated. 5 μM induce 50% of cell death [26]. 40 μM lead to 250 pg of Pt per pg of DNA, relating to 1 Pt per 1000 basepairs [27]

c) MMS is stored at 4°C as a 12 M solution, end concentration as indicated. 300 μM applications inhibit 90% of DNA synthesis [28]. 100 μM induces irreparable damage for exponentially growing cells. 200 μM induced damage can be repaired in quiescent cells [29]. MMS induces methylation of DNA by forming predominantly 3-methyladenine. This component should block DNA replication. MMS damage, induced with concentrations around 1mM, leads via Mut-genes to phosphorylation and enhanced expression of p53, at the cellular start of apoptosis induction [30,31].

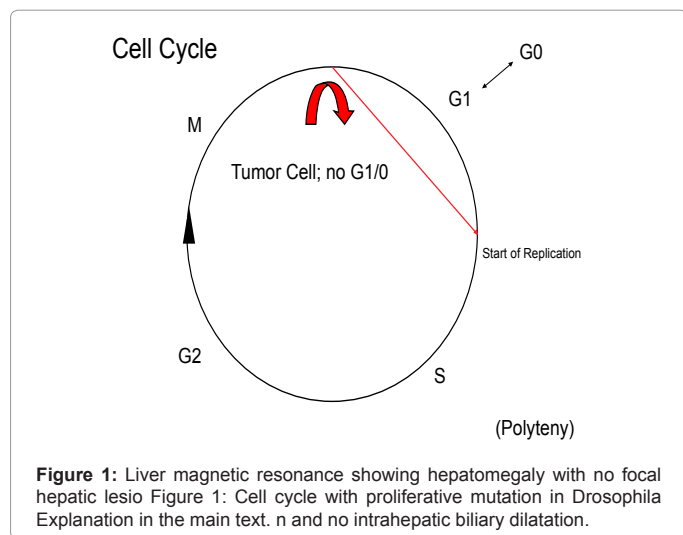
d) HU is dissolved prior to use as a 1 M solution. End concentration in experiments is 10 mM, unless otherwise stated [32]. HU is used as an antimetabolite anticancer agent. Resistance to HU may result from a mutation of the ribonucleotide reductase, or it can involve other thus far unknown mechanism [33-37]. However, it immediately inhibits semi conservative replication, whereas repair is largely unaffected [38-40]. Thus, this S-phase inhibitor can distinguish repair from replication.

e) araC is stored at -20°C as a 10 mM stock solution. End concentration is 10 mg/ml.

f) Aph is dissolved in DMSO at 1mg/ml and stored at 4°C for less than 4 weeks. End concentration in experiments is 10 μg/ml [41].

g) ddT is stored in 10 mM Tris-HCl pH 8.0 at -20°C as a 10 mM stock solution. End concentration is 100 μM [42].

Table 1: Drugs and Chemicals.



Cisplatin and Bleo for 3 days. By monitoring the cells microscopically at approximately the concentrations used in human chemotherapy, the minimal lethal dose of the chemical is determined. This is the concentration leading to lysis of more than 90% of the cells in one cell line (some cell lines are completely resistant to some drugs and show

no change in cell structure that can be evaluated by light microscopy). This dose, one lower dose, and higher doses are applied for experiments shown in figures 6,7 and table 3.

## Results

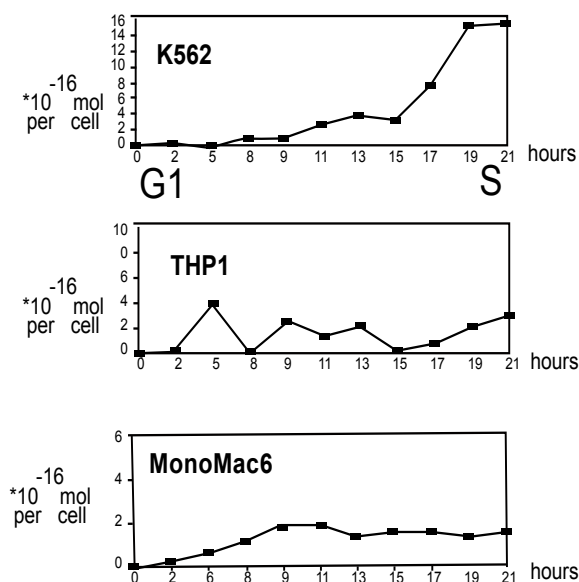
### ALL-1 tumor cells lack a G1 phase

Is the ALL-1 transformation an oncogene, tumor suppressor gene, switch gene or proliferative gene? In Drosophila, tumor formation is associated with proliferative gene defects that allow replication (Figure 1) [1]. Cells usually enter a G1 (gap 1) phase after mitosis. This is the regulation phase for each cell type, and is the time at which most biochemical functions are executed. Subsequently, the cells may enter a G0 phase, meaning that the cell reaches its final differentiation, with no remaining ability to divide. This modulating phase is shortcut by a proliferative mutation, replication, which allows replication without restriction. After the S (synthesis of DNA) phase, cells go through a short G2 phase, which cannot be shaped, but is fixed in time and biochemical action to prepare the cell division (M mitosis). Repeated replication eventually leads to polyteny, giant chromosomes, and dedifferentiates the cell, with no stopping point to arrest the tumor cell.

If the ALL-1 mutation is a proliferative gene defect, cells should allow replication. Aberrant onset of replication would be expected directly after mitosis, when G1 phase should start.

Several cell lines are synchronized with demecolcine, and *in vivo* replication is monitored by 3HT incorporation. In K562, with wild-type

### Replication Profile Over One Cell Cycle

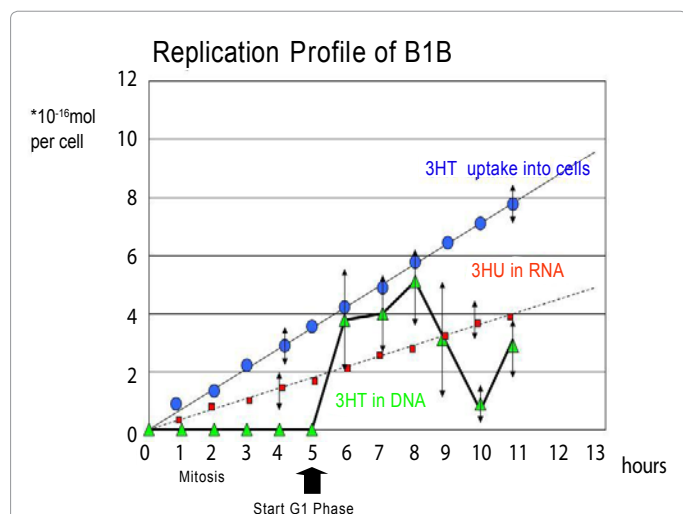


**Figure 2:** Replication Profile over one cell cycle. Cells are treated with demecolcine and released. After about 4 hours synchronous cell divisions are observed in all cases at time zero. In the experiment shown, 80% of K562, 60% of THP1, and 50% of MonoMac6 revealed microscopically visible mitotic chromosomes. Numbers are normalized to mol 3HT incorporation into DNA of one cell. Four independent samples are taken for each do. The experiment is performed once.

*ALL-1*, some DNA synthesis starts after 9-11 hours, and an S-phase (fast incorporation of 3HT into nucleic acid) occurs 15-19 hours after release of the cells (Figure 2). This is followed by a G2 phase, with no further 3HT incorporation. Cells with *ALL-1* translocations show a different profile: immediately after mitosis, 3HT incorporation starts, meaning that the replication is allowed without control. A G1 phase never occurs, and no S-phase with following G2 phase is visible, not within one cell cycle, nor within a period of 40 hours. Thus, *ALL-1* is a proliferative gene, allowing replication.

### ALL-1 tumor cells show turnover of DNA

The replication profile of THP-1 and MonoMac show instability of



**Figure 3:** Replication Profile of B1B

B1B cells are treated with demecolcine and released at time zero. Demecolcine halts the cells in mitosis, 60% of the cells revealed mitotic chromosomes. This means that 40% of the cells visible by light microscopy are possibly no longer productive (data not shown). At 4 hours, synchronous cell divisions occur for about one hour. At 5 hours cells should start their G1 phase. The blue dots line indicate the 3HT uptake into the cells, revealing continuous linear increase. The red squares indicate the incorporation of 3HU into the RNA, revealing a continuous linear increase. The green triangles indicate 3HT incorporation into DNA. Incorporation starts immediately after mitosis, thus allowing replication. Double tip arrows show the standard deviation of values. Two independent samples are taken for each result. The experiment is performed three times with similar results.

the DNA. Is this due to cell damage or is it intrinsic? Cellular damage could be seen by irregularities in 3HU incorporation. Is the instability due to irregularities in 3HT uptake rate? Irregularities could be seen, by measuring 3HT uptake. THP-1 (data not presented) and B1B cells are synchronized and DNA synthesis (3HT incorporation into nucleic acids), transcription (3HU incorporation into nucleic acids), and 3HT uptake are monitored (Figure 3). In all cases, the cellular biochemistry is stable: 3HT uptake into the cell shows a linear increase, and 3HU incorporation into RNA is continuous, therefore no damage occurred. At time zero, the cells are released from the demecolcine treatment; the mitosis takes place after about 4 hours and takes about one hour. Immediately after mitosis 3HT incorporation into DNA starts then replication is allowed. After some hours, this synthesized DNA is degraded, and another period of synthesis begins. 3HT in DNA shows turnover i.e. is incorporated and metabolized again.

A pulse of 1 hour with 3HT and a following chase without radioactivity for another hour are carried out. K562 cells, without *ALL1* transformation, do not release 3HT from their nucleic acids. All cells tested with the *ALL-1* mutation release a certain percentage of radioactivities from their DNA within one hour (Table 2). Therefore, replication is allowed by a proliferative event, and turnover of DNA is induced.

### DNA synthesis rate varies in ALL-1 tumor cells

It is expected that the replication system functions in all human cells at the same conditions at a similar rate. There is no reason to expect biochemical differences from cell to cell in this old and evolutionarily conserved biochemical system. Is the replication rate different in *ALL-1* mutant cells? This would show that these cells replicate their DNA with a different system. Quantitative DNA synthesis is monitored in unsynchronized tumor cells. K562 show incorporation of around twice as much DNA per cell per hour than cells with the *ALL-1* translocation (Table 2). The synthesis rate of THP1 and B1B is significantly lower, meaning these cells incorporate less than half of the 3HT stably to their DNA per hour. Interestingly the long-term incorporation rate per nucleic acid content does not reflect this finding. It is expected that the radioactivity per  $\mu\text{g}$  of nucleic acid is highest in K562 and lower in B1B and THP1 cells. This is correct for B1B: the stably incorporated 3HT is five times lower than in K562. But THP1 and MonoMac6 incorporate long term 10 times more radioactivity into their DNA. This indicates that different systems are induced by the transformation proteins.

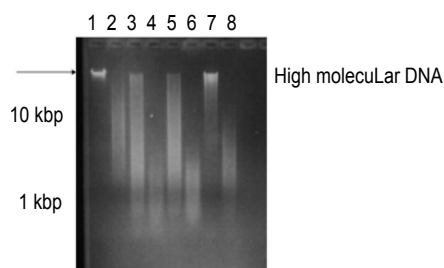
	U937	Nalm6	K562	B1B	SEM	RS4:11	MV4:11	ML2	MMac6	THP1
<i>ALL-1</i> a)	-	-	-	4;11	4;11	4;11	4;11	6;11	9;11	9;11
Release of 3HT b) [%]	3	n.d	0	3	n.d.	9	14	3	4	n.d
3HT incorporation to DNA per hour per cell [x 10 <sup>-17</sup> M] c)	n.d	n.d	7.5	3.0	n.d	n.d	n.d	n.d	n.d	4.0
3HT incorporation rate d) long term [relative]	222	129	254	55	25	42	7	57	470	515
Cellular response to HU e)	S	S	R	T	n.d.	T	T	T	S	S
Effect of HU f)	0,8	1,6	1,9	0,9	0,9	1,0	1,4	1,5	1,2	1,8

- a) Translocational aberration of cell line  
 b) Pulse and chase experiment. Unsynchronized cells are incubated with 3HT for 1 hour and chased for another hour. Loss of radioactivity from DNA is indicated in percent.  
 c) Unsynchronized cells are incubated with 3HT, and the incorporation rate into DNA per cell per hour is determined.  
 d) Cells are incubated with 3HT for 19 hours. Nucleic acids are isolated, and the relative incorporation rate of radioactivity per nucleic acid content is determined.  
 e) Resistance (R), Tolerance (T) or Sensitivity (S) after incubation with HU for three days monitoring cell count and cell survival by microscope. S means no surviving cell, T means decrease of cell number with at least 10% remaining cells, R means cell divisions occur (Results from two experiments).  
 f) Cells are incubated for 19 hours with or without HU; nucleic acids are isolated and quantified. The quotient of the DNA content per sample without/with HU is indicated. 1 means the same amount with and without HU. 2 mean duplication of the DNA without HU and arrest of DNA replication with HU.  
 Each number reflects the average of two samples per experiment, with two independent experiments unless indicated otherwise.  
 n.d. not determined

**Table 2:** Response of Cells.



### DNA Structure of Leukaemic Cell Lines



**Figure 4:** DNA is fragmented in *ALL-1* tumor cells  
Agarose gel electrophoresis (1%) of cell lines stained with ethidium-bromide. Each DNA sample is shown before heat denaturation and after heat denaturation. Nalm6 (lines 1, 2) and U937 (lines 7,8) DNA is high molecular and less fragmented than DNA from mutant *ALL-1* bearing cells B1B (lines 3,4) and ML-2 (lines 5,6).

T(9;11) induces activity of different enzymes with different binding affinities than T(4;11). These results show that *ALL-1* mutations induce replication and they replicate their DNA with different systems.

### DNA is fragmented in *ALL-1* tumor cells

Is the genomic DNA in *ALL-1* mutants normal? Ongoing DNA turnover could lead to genome instabilities and to elevated levels of fragmented single stranded DNA. Carefully isolated DNA from normal cells agglomerates into filaments when ethanol for precipitation is added, resulting in long DNA strands. Usually these long filaments dissolve slowly. It is observed, that DNA from tumor cells behaves differently, never agglomerating to filaments and dissolving easily after precipitation. Figure 4 shows native DNA and heat-denatured single strand fragments of tumor cells. Whereas U937 and Nalm6 show some amount of high molecular DNA, native DNA of *ALL-1* mutant cell lines is degraded down to about 1,000 base pairs per fragment. U927 and Nalm6 show 1,000 to 10,000 bases per single stranded fragment. *ALL-1* mutant cells have DNA single stranded fragments only 20 to 2,000 bases long (Figure 4). Additionally all cell lines without *ALL-1* transformation (U937, Nalm6 and K562) show occurrence of satellite DNA in agarose gel electrophoresis, but ML-2 and B1B do not (data not shown). Therefore, the *ALL-1* mutants show differences in DNA structure.

### Constant onset of the repair system in *ALL-1* tumor cells

If DNA is continuously metabolized in *ALL-1* mutants, and the kinetics of the replication systems is different, and the DNA is more fragmented, what is activity of the repair system? Hydroxyurea (HU) inhibits semi-conservative replication (it inhibits the S-phase) whereas unscheduled DNA synthesis (i.e. repair mechanisms) is not affected. Incubation of cells without or with HU occurs for 18 hours, and the content of DNA is quantified (Table 2). K562 duplicates the genome without HU during this time, and HU inhibits completely this DNA synthesis. However, the cells themselves appear to be resistant and show no apoptosis. B1B, SEM, RS4; 11, MV4; 11, ML2, MonoMac6, and THP1 cells show few effects of HU on their DNA-synthesis. B1B and SEM even produce more DNA under HU. 3HU incorporation is not affected, thus cells are not stressed during the treatment, and however, they show some cellular damage over the long term (Table 2). Resistance of the DNA synthesis to HU in *ALL-1* mutant cell lines show constant onset of the repair system in these cells. ddT inhibits repair synthesis and allows replication synthesis. Thus, HU

and ddT complement each other, and together should reduce all DNA synthesis to zero.

Cells are incubated with either HU, or ddT, or both drugs, and DNA synthesis is monitored by 3HT incorporation. K562 cells show HU-sensitive DNA synthesis, thus they replicate DNA in a semi-conservative way. Replication in B1B, SEM, ML2 and Monomac6 is about 60% HU sensitive and ddT resistant and about 40% HU resistant and ddT sensitive. Experiments with AraC and Aph lead to similar conclusions (data not presented). Thus, the DNA repair system is constantly active to a large extent in *ALL-1* transformed cells.

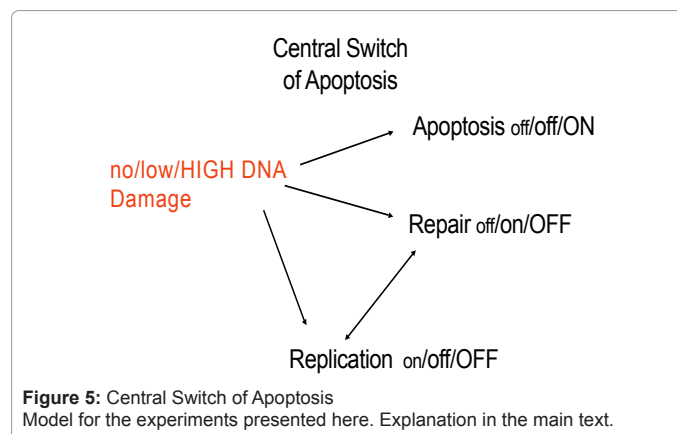
### Central switch to apoptosis

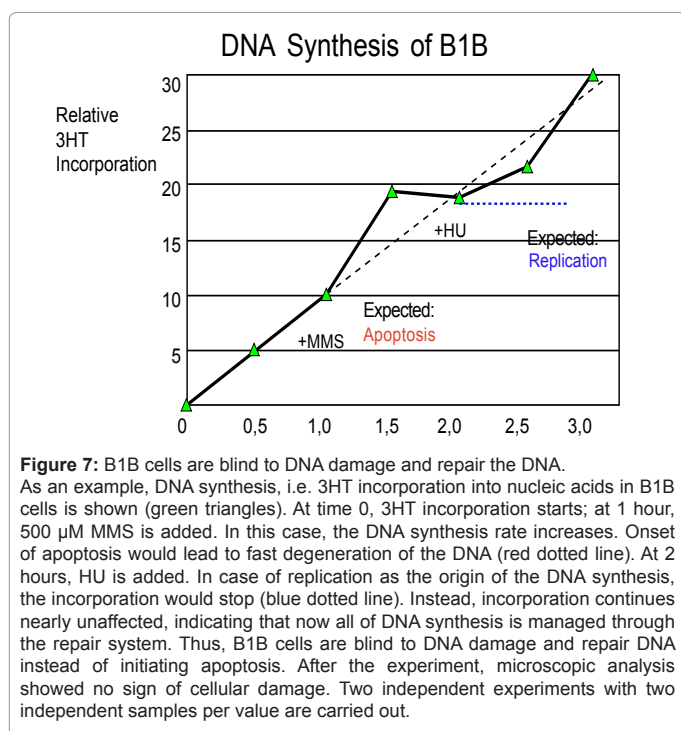
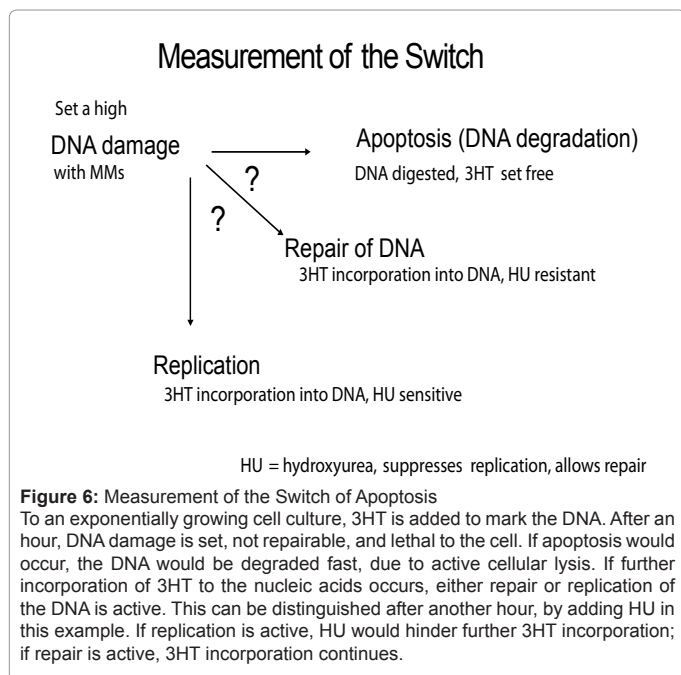
*ALL-1* mutation allows aberrant replication, induces repair synthesis. How do the cells react upon DNA damage to induce apoptosis? Figure 5 outlines the experimental model of the central switch of a cell to regulate the replication system, and keep the genome intact. If there is no DNA damage, apoptosis should not be necessary; the repair system is off and replication is possible. If there is low, i.e., repairable DNA damage, apoptosis is off. One signal should stop replication to avoid establishment of mutations. One signal should induce, possibly via p53, the repair system [24]. If repair is successful, offset of repair and onset of replication follow. If severe DNA-damage is induced, like a number of double strand breaks of both homologous chromosomes that physically hinder recombination repair, replication and repair should stop. One signal to p53 should raise the apoptotic cascade. Herein, DNA damage leads the process, and a restriction point excludes both repair and replication at the same time.

One of the possibilities to measure this switch is shown in figure 6. If a high (lethal) DNA damage is set, apoptosis (fast degradation of the DNA) should take place. Replication or repair should switch off. In case of DNA synthesis, both can be distinguished with the addition of HU.

DNA-damaging agents like MMS, Cisplatin or Bleo induce apoptosis in normal cells. (Figure 7) shows one way to measure the response to DNA damage of B1B cells. These are blind to DNA damage and show HU-resistant DNA synthesis instead, i.e., they repair their DNA. A number of different concentrations of MMS, Cisplatin and Bleo with leukemic cell lines lead to following results (Table 3).

**DNA degradation rarely occurs:** Only SEM cells degrade 20-50% of their DNA upon addition of Bleo or Cisplatin, all other cells are resistant to these drugs.





- Only SEM cells degrade some of the DNA upon addition of MMS, all other cells are resistant to this drug.

In no case, even with destructive concentrations of one of these drugs, can apoptosis be induced in ALL-1 mutants.

**DNA synthesis is rarely affected:** Only SEM cells stop replication when Bleo or Cisplatin are administered.

- All cells are blind to MMS-induced damage and continue to synthesize DNA at nearly normal rates.

Even with the lethal doses of DNA-damaging agents, all cells induce HU resistant DNA synthesis, i.e. repair DNA at some point.

Altogether, only partial DNA degradation occurs in SEM cells upon application of lethal doses of DNA-damaging drugs. Thus, all cells cannot react accordingly. DNA synthesis continues in most cases. This DNA synthesis is partially resistant to HU, thus the tumor cells replicate and repair DNA rather than dying. This is summarized in figure 8 in red: the proliferative mutation allows replication, the repair system is induced. In that, cells cannot react to DNA damage and thus, apoptosis cannot be switched on.

## Discussion

Tumor formation is similar in humans and in *Drosophila*. The biochemical event in tumor initiation is a proliferative mutation that

	U937	K562	B1B	SEM	ML2	MonoMac6
<b>MMS 500 μM (lethal) a)</b>	T	R	T	T	T	R
Stop DNA Synthesis b)	--	--	--	-	-	--
Onset Repair c)	-	0	+	+	+	0
DNA degradation d)	0	-	-	0	-	-
<b>Cisplatin 500 μM (lethal) a)</b>	T	R	T	T	S	R
Stop DNA Synthesis b)	0	-	--	++ e)	0	-
Onset Repair c)	-	+	+	-	-	+
DNA degradation d)	0	-	-	+	+	-
<b>Bleo 0.1U/ml (lethal) a)</b>	T	T	T	T	S	S
Stop DNA Synthesis b)	--	-	-	++ e)	-	-
Onset Repair c)	-	0	+	0	+	-
DNA degradation d)	-	0	-	++	-	0
<b>Cisplatin 50 μM (sublethal)</b>						
Stop DNA Synthesis b)	+	--	--	++	0	-
Onset Repair c)	0	0	+	-	+	-
DNA degradation d)	0	-	-	++	0	0
<b>MMS 100 μM (sublethal)</b>						
Stop DNA Synthesis b)	-	--	--	+	--	0
Onset Repair c)	+	0	+	+	-	+
DNA degradation d)	0	-	-	+	-	0
<b>MMS 12 mM (destructive)</b>						
Stop DNA Synthesis b)	+	0	--	+	+	0
Onset Repair c)	-	0	-	+	-	0
DNA degradation d)	++	0	+	0	+	0

a) Cellular response. Cells are incubated for three days with the drug indicated; cell viability and cell count are monitored by light microscopy. Sensitivity (S, no surviving cell), tolerance (T, decrease of cell number, at least 10% surviving cells) and resistance (R, increase of cell number) is distinguished. Results from two experiments. Following data are obtained by monitoring 3HT incorporation in similar experiments as outlined in figure 7.

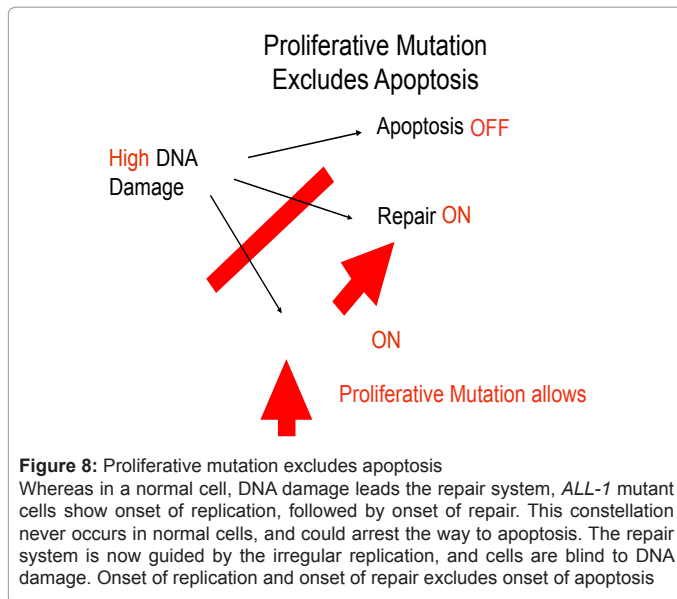
b) After one hour of 3HT incorporation, the drug (MMS, Cisplatin, or Bleo) is added. Definitions are: no further increase (+); release of radioactivity (++); further 3HT incorporation with reduced rate (-); no influence on DNA-synthesis rate (--).

c) One hour after step b), HU is added. In case of active repair synthesis, 3HT incorporation is not affected. 10-100% resistance (+); less than 10% resistance (0); release of radioactivity (-) can be distinguished. Similar experiments with ddT instead of HU are carried out, leading to similar conclusions (data not shown).

d) After step c) two hours of 3HT incorporation are monitored. Definitions are: loss of more than 40% of the radionuclide in nucleic acid (++); loss of more than 20% (+); steady state of DNA, losing between 10% and 20% (0); DNA synthesis (-).

e) Despite DNA degradation of SEM cells, no apoptosis takes place, the cells remain intact. At the end of the incorporation experiment, cells are analysed by microscopy. All cells remain physically intact after the four hours of incubation. The experiments are outlined twice with two independent samples per point.

**Table 3: Switch to Apoptosis.**



allows replication, inducing the shortcut to S-phase. In *ALL-1* mutants, replication starts immediately after mitosis, thus, replication is allowed by the tumor-inducing mutation. This defines *ALL-1* as a proliferative gene. This finding is novel. It is the first proliferative gene identified in human cells. In molecular terms, *ALL-1* shows AT-hooks, that are able to bind to origins [11] and methyltransferase activity that affects chromatin structure. In *Drosophila*, proliferative mutations induce fluffy chromosomes, which are only partially packed and contain somatic pairing gaps.

The repair system is involved in tumor formation. Here, the involvement of the repair system in tumor cells is shown:

All of the *ALL-1* mutants show instable 3HT incorporation into the DNA. After some hours, the synthesized DNA is metabolized, indicating turnover of DNA. Cells without *ALL-1* mutation do not show instability of DNA. The repair system is continuously on in *ALL-1* mutants. Different kinetics of the DNA synthesis in different cell lines point towards usage of different enzymes or systems. Possibly different transforming proteins induce replication at different biochemical corners.

A strong tumor-inducing gene, which initiates DNA replication, could initiate the same process several times during one cell cycle at the same origins of replication. By mutation of proliferative gene *bellatrix* in *Drosophila*, one homologous chromosome is over-replicated, whereas the partner chromosome replicates to a lower copy number, leaving the total copy number of a region constant [2]. Thus, repetitive initiation of replication at one point on one chromosome could be followed by copy number control, repair, and reduce of copy number by DNA degradation. This could then cause a certain turnover of DNA, as is observed here in *ALL-1* mutants. And repair replication is continuously active in *ALL-1* tumor cells.

Many tumor cells cannot induce apoptosis. Induced lethal DNA damage should stop replication and stop repair of DNA but induce apoptosis. Induced low DNA damage should stop replication and induce repair of DNA. Neither response can be measured in leukemic cell lines. It is concluded that leukemic cells are blind to DNA damage and replicate or repair their DNA without control, rather than undergoing apoptosis. Thus, they show an intrinsic resistance to DNA

damage-inducing chemotherapy. They are in a condition of apoptosis defect.

In normal cells, the DNA damage leads the way to onset either repair or apoptosis. In normal conditions, either the repair system is on or the apoptosis starts. Replication and repair are never initiated at the same time. Under normal conditions, replication during a repair period would manifest mutations. Proliferative genes allow replication, followed by onset of DNA repair. This never occurs in normal cells and this onset of both systems could block the way to apoptosis completely.

On the background presented here, chemotherapy should be questioned. HU is used as a drug in tumor therapy. Allowing repair synthesis, it will select cells that “replicate” their DNA with repair synthesis. As this process is more or less constitutive in possibly any tumor cell, all tumor cells are intrinsically somewhat resistant to HU. In contrast, ddT might be a drug of choice for initial chemotherapy, because it inhibits polymerase beta, an enzyme needed in repair replication, that is needed in tumor cells but not required for cell survival of normal cells. It will inhibit tumor cells to some extent, but not induce apoptosis. Use of DNA-damaging agents has to be questioned on this background as well. Any tumor cell line analyzed, as well those lines without *ALL-1* translocations, show some resistance to these drugs. If it is not possible to induce apoptosis, surviving cells grow with additional mutations, and with more aggressive tumor growth.

Instead, a successful tumor therapy should not focus on apoptosis of cells, but instead seek to inhibit the activity of tumor cells. Damage to the immune system should be avoided. Immune cells can recognize tumor cells and can lyse them, as with the Amanita therapy [25].

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