CONSEQUENCES OF INTERSTRAND CROSSLINKS IN HEMATOPOIESIS:

TIPPING THE BALANCE BETWEEN SENESCENCE AND PROLIFERATION

Judith Verhagen-Oldenampsen

Consequences of Interstrand Crosslinks in Hematopoiesis:
Tipping the balance between senescence and proliferation

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Auteur: Judith Hubertina Elisabeth Verhagen-Oldenampsen
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Consequences of Interstrand Crosslinks in Hematopoiesis: Tipping the balance between senescence and proliferation

Consequenties van interstrand crosslinks in hematopoiese: Verschuivingen in de balans tussen veroudering en proliferatie

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I was up, I was down,
I felt almost everything.
I was lost, been around,
still know hardly anything.
Get my feet on the ground
and the heart of everything.
It's just the way that I want it.

Tonight's the night,
time to leave the pressure behind.
There's no surrender.

Voor mijn familie

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1

General Introduction

1 HEMATOPOIESIS

1.1 The hematopoietic system

Hematopoiesis is the process of blood cell formation, schematically depicted in Figure 1. Each day, an adult produces around 10^{11}-10^{12} new blood cells. All blood cells are derived from hematopoietic stem cells (HSC) that reside in the bone marrow (BM). HSCs have the capacity to either self-renew or give rise to daughter cells with limited renewal capacity. These cells are indicated as long term repopulating stem cells (LT-HSC) and short term repopulating stem cells (ST-HSC), respectively. The ST-HSC differentiate into multi-potent progenitor cells (MPPs) that undergo transient amplification and contribute largely to the quantitative output of blood cells from the BM. In the hierarchical model of hematopoiesis, MPPs give rise to common myeloid progenitors (CMPs) and common lymphoid progenitors (CLPs), probably through a stochastic mechanism in which survival is limited by the growth factors that are present. In turn the CMPs and CLPs give rise to the committed cells of the myeloid and lymphoid lineages [1-3]. Whereas the CLPs produce both T-, B-, and NK- cells, the CMPs give rise to macrophages, granulocytes, erythrocytes and platelets (Figure 1). Next to this classical model of hematopoiesis an alternative model exists in which lymphoid primed MPPs (LMPPs) exist which have both lymphoid and myeloid differentiation potential but lack erythro-megakaryocyte potential [4]. The identification of these LMPPs suggests that at least the granulocyte-macrophage lineage can be generated through an alternative mechanism.

The balance between proliferation, differentiation and cell death is tightly regulated in hematopoiesis. Disruption of this regulation can lead to a survival advantage or an increased replicative potential, which can result in leukemia [5]. For example, human chronic myeloid leukemia (CML) is a two stage disease of the BM which is caused by chromosomal translocation t(9;22). Due to this translocation the BCR-ABL protein is produced. Initially this will lead to hyperproliferation of white blood cells, which is the direct result of the constitutive kinase activity of BCR–ABL and activation of signal transduction pathways. Further mutations can induce the second stage of CML (blast crisis), that is characterized by impaired differentiation capacity and an increased number of blast cells in the circulation [6].

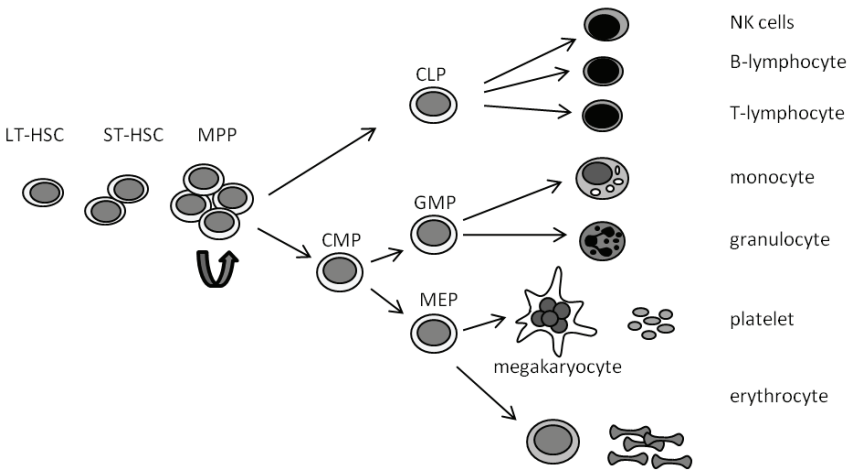

Figure 1: Simplified scheme representing hematopoiesis. For details see text.

Acute myeloid leukemia (AML) is a heterogeneous group of leukemias in which the majority of the leukemic cells get stuck in an immature blast cell stage, representing precursors of early myeloid, erythroid, megakaryocytic, granulocytic, or monocytic cell lineages. The prognosis for patients with AML is mainly dependent on the genetics of the neoplastic cells [7]. The molecular markers with prognostic impact include recurrent translocations, mutations in FLT3, NPM1, MLL, WT1, c-KIT, and expression levels of BAALC, NM1, ERG, and CXCR4 (reviewed in [8]). Classification of these different AML subtypes using molecular markers is relevant because specific abnormalities are associated with clinical behavior. Favorable karyotypes with usually a good response to therapy include: t(8;21), inv(16) and t(15;17). In contrast, poor response is associated with for instance inv(3), or with a normal or complex karyotype. Therapy-related AML and AML arising from myeloid dysplastic syndrome (MDS) are also associated with poor response to therapy. While advances have been made in the understanding of AML, characterized by recurrent translocations such as t(8;21) and t(15;17), our understanding of poor response leukemia subtypes is still limiting and requires future research.

Bone marrow failure is characterized by impaired hematopoiesis and some BM failure syndromes associate with cancer predisposition [9]. It occurs most frequently in the elderly population, and at low frequency as congenital BM failure syndromes. BM failure occurs more frequently in the East than in the West [10]. In Japan and the Far East, the frequency is at least 3 times higher than it is in the United States and Europe, even after correction for ethnic differences. It has been suggested that environmental factors and persistent use of insecticides contribute to the cause of the disease [11].

Although congenital BM failures are rare, the molecular characterization of these syndromes may give insight into BM failure in general. The most frequent congenital syndromes are described in more detail in the following sections.

1.2 Congenital Bone Marrow Failure Syndromes

1.2.1 Diamond-Blackfan anemia (DBA)

Diamond-Blackfan anemia (DBA) is a congenital aplasia in which specifically the erythroid compartment is compromised. DBA is a disorder of ribosome biogenesis. To date 11 different structural ribosomal proteins of both the small and the large ribosomal subunit were found to be affected, with mutations in Rps19 being the most frequent aberration [12]. DBA usually becomes apparent during the first year of life. Only occasionally it evolves into an aplastic anemia or leukemia. DBA is associated with craniofacial and thumb abnormalities as well as short stature. Cardiac defects are seen in one third of DBA patients. DBA patients are at risk of developing solid tumors and hematological malignancies later in life, which becomes apparent now that better treatment improves the life expectancy of DBA patients [13].

1.2.2 Shwachmann-Diamond syndrome (SDS)

Shwachmann-Diamond syndrome (SDS) is an autosomal recessive disorder. Around 90% of patients carry a mutation in the SBDS gene that encodes an enzyme involved in the release of the 60S ribosomal subunit from the nuclear-cytoplasmic transport complex. [14]. Failure to release the 60S subunit impairs association of the small and large ribosomal subunits and inhibits translation initiation. It presents in childhood and is characterized by neutropenia that progresses to BM failure. Patients may also suffer from pancreatic failure and skeletal abnormalities [15]. SDS is associated with a high tendency towards malignant myeloid transformation into myelodysplastic syndrome and leukemia [16]. However solid tumors are a rare event in SDS, maybe because the patients succumb to the disease before solid tumors arise.

1.2.3 Dyskeratosis Congenita (DC)

Dyskeratosis Congenita (DC) is a congenital BM failure that is characterized by abnormal skin pigmentation, nail dystrophy and mucosal leukoplakia [17]. Several subtypes of DC exist: X-linked recessive, autosomal dominant and autosomal recessive. BM failure is the major cause of mortality with an additional predisposition to malignancy and fatal pulmonary complications. Most genes that are affected in DC affect a protein complex involved in uridylation of ribosomal RNA and the RNA component of the telomerase complex [18]. Other genes associated with the disease encode for proteins active in the telomere complex [19]. To what extent telomerase dysfunction and ribosome maturation contribute to this disease is still subject to controversy [20].

1.2.4 Fanconi Anemia (FA)

FA patients often present with a combination of several congenital abnormalities including short stature and skin hyperpigmentation [21]. Most of the patients that are diagnosed with FA will develop BM failure during the first or second decade of life. There is a strong predisposition for both solid tumors and hematological malignancies such as MDS or AML [22]. Currently 15 FANC genes have been identified that contribute to the disease [23-24]. The corresponding proteins function in a DNA repair pathway that is also indicated as the Fanconi pathway and is involved in DNA repair during DNA replication. At the cellular level, the hallmark phenotype of FA cells is pronounced hypersensitivity to agents that produce DNA interstrand crosslinks (ICLs) such as mitomycin C, diepoxybutane, cisplatin, nitrogen mustard, melphalan, cyclophosphamide and furocoumarins in combination with ultraviolet (UV) radiation exposure [25].

The clinical variability in congenital abnormalities and the progressive BM failure characteristics of FA patients is high [26], which is only partially linked to the different genotypes among these patients [27]. Mosaicism in the hematopoietic system contributes to this phenomenon [28]. In some patients with different mutations on the two alleles of a *FA* gene, one of the 2 mutated FA alleles may revert to its wildtype configuration through homologous recombination between the alleles in a hematopoietic stem cell. These reverted cells have a selective growth advantage over cells carrying 2 mutated FA alleles, and the reverted cells repopulate the BM. By consequence, patients have normal blood

counts, while they still have an increased risk of FA-related cancers in tissues that still carry two mutated alleles. This mechanism resembles the strong competitive advantage of non-recombined hematopoietic cells in mice carrying a conditional *Ercc1* allele (Chapter 3).

1.2.5 Severe Congenital Neutropenia (SCN)

SCN represents a heterogeneous group of inherited disorders that are characterized by low neutrophil counts. It is a preleukemic condition independent of its genetic subtype. The acquired mutations in the G-CSF receptor (CSF3R) are specific for SCN and are strongly associated with malignant progression. Several causal gene mutations have been identified in SCN, such as genes encoding chaperones or proteins involved in the unfolded protein response; *ELANE [29]*, *GFI1 [30]* and *HAX1* [29]. The clinical diagnosis is usually made shortly after birth or during the first months of life, based on recurrent severe infections and severely reduced neutrophil count [31]. For most subtypes the underlying mechanism is considered to be increased apoptosis of neutrophils and neutrophil precursors [32-33].

In summary, the congenital BM failure syndromes affect ribosome synthesis, posttranslational quality control of proteins, telomere protection and DNA repair. Defects in these processes are sensed by proliferating cells and will lead to the activation of tumor suppressor systems with the aim to prevent wasting energy on ribogenesis, or permanent DNA damage [34]. The activation of tumor suppressor genes may explain the BM failure. The selection for cells that escape this repression and which are then able to repopulate the BM, may contribute to the subsequent risk for leukemic derangement (reviewed in [35]). Zebrafish and mouse models were generated for DBA and DC that recapitulate the disease. Notably, the disease phenotype is abrogated upon deletion of the tumor suppressor gene p53 [36-37] (see also section 3 about tumor suppressor genes and the DNA damage response).

1.3 Acquired Bone Marrow failure

1.3.1 Acquired Aplastic Anemia (Acquired AA)

Aplastic Anemia results from a marked decline of blood cell production by the BM. As a result reticulocytopenia, anemia, granulocytopenia, monocytopenia and thrombocytopenia arise [38]. Most cases of AA are acquired while only a small percentage of patients with AA are the result of an inherited disorder, such as Fanconi Anemia and Shwachmann-Diamond syndrome. The known causes of acquired AA include autoimmune disease [39], but also prolonged exposure to high doses of toxic chemicals (e.g. benzene) [40], viral infections (e.g. Epstein-Barr) [41], and distinct reactions to certain pharmaceuticals (e.g. chloramphenicol) [42-43]. In the latter conditions, acquired AA is most likely caused by the activation of tumor suppressor genes.

1.3.2 Myelodysplastic syndrome (MDS)

MDS is a HSC disorder characterized by ineffective hematopoiesis, various degrees of cytopenia and dysplastic features of peripheral blood and BM cells. MDS is distinctly different from acquired AA because the BM is not severely hypoplastic. Instead there may even be an excess of blast cells, in which case, mutations in these blasts indicate clonal expansion of BM progenitors.

Although MDS is not considered to be a BM failure, there are similarities between congenital BM failure and MDS. For instance 5q- MDS includes deletion of ribosomal protein RPS14, which suggests that there may be similarities between 5q- MDS and DBA. Furthermore, monosomy 7 is a common aberration found when MDS or FA BM undergoes leukemic transformation [44].

Whereas Fanconi Anemia is due to impaired DNA repair, a subgroup of MDS may result from exposure to DNA damaging drugs. This may be particularly true for patients that were previously treated for cancer [45-46]. For example, patients treated for breast cancer have a 3.5 fold increased risk for MDS development [47]. Therapeutic agents which are known to predispose for MDS include alkylating agents, topoisomerase II inhibitors and epipodophyllotoxins. Also radiation therapy increases the risk of MDS and leukemia development.

Patients who suffer from MDS, AA or BM failure syndromes are at increased risk to develop leukemia. Three possible hypotheses are proposed to explain clonal evolution in the context of BM failure. 1) Acquisition of a genetic defect by one individual HSC can result in outgrowth of a leukemic clone via a mechanism independent from the underlying MDS/BM failure syndrome. 2) The cytopenia in BM and/or peripheral blood may select for a genetic defect that specifically overcomes the action of tumor suppressor genes. These cells will have a proliferative advantage and cause monoclonal hematopoiesis. 3) The BM senescence induced by tumor suppressor genes may induce epigenetic changes that enable some HSC to proliferate and repopulate the BM. Selection for the epigenetic changes that give the best adaptation will give rise to clonal hematopoiesis although DNA markers will not be present.

A comparison of MDS transforming to AML with congenital BM failures that undergo leukemic transformation suggests that at least part of the MDS patients may suffer from a BM failure in which mutations give proliferative advantage of the transformed cells over the affected cells in the population [48-49].

While several genes and chromosomal regions have been associated with MDS, a commonly deleted tumor suppressor gene has not been identified. It is possible that haploinsufficiency and reduced gene dosage for critical genes involved in hematopoiesis are located on chromosome 5q and 7q, and may sufficiently alter the balance between growth and differentiation to induce dysplastic hematopoiesis. Alternatively, epigenetic inactivation of the remaining allele or alterations in gene expression through loss of microRNA (miRNA) loci could also play a role.

In this thesis we investigate the role of DNA damage and impaired repair in BM failure. Currently it is still largely unknown how DNA damage affects the hematopoietic system, and which DNA damage-induced tumor suppressor genes are involved in BM failure and subsequent leukemic progression. A model in which the effect of distinct levels of DNA damage on the hematopoietic system can be studied may enable the elucidation of specific molecular pathways that are involved in the transition from a damaged BM environment to leukemia.

2 THE DNA DAMAGE RESPONSE

2.1 DNA damage and its consequences

The genome of every living organism is continuously damaged by endogenous and exogenous agents that modify the integrity of the DNA. Thereby the genetic information of the DNA is constantly challenged. DNA damage is an undesired chemical alteration in the base, sugar or phosphate group that alters the properties of the DNA helix. In contrast to mutations, that are fixed errors in the coding information of the genome, DNA damage can be repaired by specialized mechanisms.

DNA damage can be induced by exogenous physical agents, endogenous chemical genotoxic agents, which are byproducts of metabolism such as reactive oxygen species (ROS), or by spontaneous chemical reactions such as hydrolysis. The main types of damage, their sources and main repair mechanisms are listed in Table 1.

Table 1: Types and sources of DNA damage

Type of DNA damage	Major source of DNA damage	DNA damage repair mechanisms*
Abasic site	Spontaneous or alkylation induced	BER
8-oxo-guanine (8-oxo-G)	Reactive oxygen species (ROS)	BER
6-4-photoproduct ((6-4))-PP)	UV light	NER
Cyclobutane pyrimidine dimer (CPD)	UV light	NER
Single strand break (SSB)	Reactive oxygen species (ROS)	NHEJ, HR, BER
Double strand break (DSB)	Ionizing radiation (IR)	NHEJ, HR
Mismatch (MM)	DNA polymerase infidelity	MMR
DNA-protein crosslink	DNA topoisomerase enzymatic reaction	Direct reversal, NER, HR
Interstrand crosslink (ICL)	Bi-functional alkylating agents/UV light/IR	NHEJ, HR

* BER: Base Exchange Repair, NER: Nucleotide Excision Repair, NHEJ: Non-Homologous End-Joining, HR: Homologous Recombination, MMR: Mismatch Repair.

2.1.1 Types of DNA damage

The type and extent of DNA damage determines the fate of damaged cells. Some lesions are mainly mutagenic (oxidative lesions such as 8-oxoG), whereas others are more cytostatic or cytotoxic (double strand breaks and interstrand crosslinks). Most lesions however will have both mutagenic and cytotoxic properties varying in ratio depending on location and number of lesions as well as cell type, cell cycle stage and differentiation status.

Mutagenic lesions are usually small changes to the base pairing region of DNA bases, including spontaneous deamination, depurination and oxidation of bases [50]. These lesions, if left unrepaired, will not block DNA metabolic processes such as replication but they influence replication fidelity. Thereby largely increasing the risk of mutations which in turn may contribute to carcinogenesis [51]. However larger helix distorting lesions (e.g UV-induced pyrimidin dimers) which do block replication, can also be carcinogenic when left unrepaired.

Cytotoxic lesions are chemically diverse, including crosslinks and double strand breaks (DSBs). Unrepaired cytotoxic lesions block metabolic processes in the cell, ultimately leading to proliferative arrest or cell death. The cumulative effect of cytotoxic lesions is therefore very likely to be a decrease in functional or proliferative capacity of cells, resulting in aging features in the affected tissues or organs.

2.1.2 Consequences of DNA damage

When damage is inflicted in actively transcribed genes it will block transcription and affect protein synthesis. If mutations occur in the DNA due to error-prone repair or faulty replication, these will irreversibly change the genetic make-up of the cell. These mutations, as well as other changes in the DNA resulting from genome instability or miss-segregation (chromosome rearrangements, deletions, insertions, duplications and loss of heterozygosity) could lead to cancer, inborn defects and overall malfunctioning of cells. Consequently, genetic damage has both immediate as well as long term effects.

To protect the genome from the deleterious effects of DNA damage, cells are equipped with an arsenal of complementary DNA repair pathways ([52], Table 1). In addition, cells are able to temporarily block cell cycle progression to allow repair before damage is converted in permanent mutations during replication [53]. Finally cells may use alternative pathways such as apoptosis [54] or senescence [55] when damage levels are too

high. The next sections of this introduction will focus on the mechanisms that are specifically related to hematological defects and bone marrow failure syndromes. These include DNA damage checkpoints (section 2.2), double strand break (DSB) repair (section 2.3), nucleotide excision repair (NER) (section 2.4) and interstrand crosslink (ICL) repair (section 2.5).

2.2 DNA damage checkpoints

Maintaining genomic stability is of vital importance to cells. Any change to the genomic structure can lead to uncontrollable proliferation or cell death. To ensure correct genetic transfer throughout generations, cells have a set of surveillance and DNA repair mechanisms that prevent damaged DNA from being converted into heritable mutations. These surveillance mechanisms are called DNA damage checkpoints. They act as signal transduction pathways triggered by DNA damage and will result in inhibition of cell cycle progression and/or DNA replication (Figure 2, [56]). They also induce efficient removal of lesions and an increased resistance to future lesions [57]. At the top of the checkpoint cascades are two protein kinases of the PIKK family, Ataxia Telangiectasia mutated (ATM) kinase and ATM and Rad3 related (ATR) kinase. ATM responds mostly to double strand breaks while ATR responds to replication stress, but both respond to a variety of other DNA lesions [58]. There is also crosstalk between these two pathways, for instance because single strand breaks (SSBs) can become DSBs when stalled replication forks collapse.

Upon DSB induction, the MRN complex consisting of Mre11, Rad50 and Nbs1 binds to the DSB [59]. The MRN-DSB complex recruits ATM to the breakage site. The exact mechanism of ATM activation is still not clear, there are several proposed mechanisms such as autophosphorylation of ATM [60], activation via the MRN complex [61] and chromatin alterations [60]. Upon interaction of ATM with the MRN complex, other DNA damage response proteins are recruited to the DSB site. These DNA damage response proteins include (among others); MDC1, 53BP1 and BRCA1. Simultaneously ATM phosphorylates core histon 2AX (H2AX; referred to as γH2AX when phosphorylated), this histone is part of the nucleosome structure around which the DNA is wrapped. γH2AX is believed to stabilize the interaction of DSB response proteins at the breakage site and serves as a platform to facilitate accumulation of DNA repair proteins onto the damaged DNA [62]. ATM also phosphorylates and activates Chk2 which in turn phosphorylates and stabilises the tumor suppressor protein p53. The increase in p53 inhibits cell cycle progression (via p21/Cyclins) and induces

apoptosis (via PUMA/BAX/NOXA). Failure to repair DSBs prolongs the activation of ATM. For cells that are in G1 or S-phase of the cell cycle this will result in a G1 arrest due to activation of the cell cycle checkpoint kinase Chk2. If cells are in the G2 phase of the cell cycle Chk1 will be activated, resulting in mitotic arrest.

ATR can be activated by many different types of damage, including stalled replication forks. The immediate consequence of stalled replication forks is an increase in single stranded DNA (ssDNA), which is the primary signal for ATR activation [63]. The ssDNA is coated by RPA, and this complex recruits the ATR interacting protein (ATRIP) which is constitutively bound to ATR [64]. The binding of the ATRIP-ATR complex to the ssDNA-RPA site does not yet activate ATR. This is done by TopBP1 which is recruited to the ssDNA-RPA site by the 9-1-1 complex [65]. Activated ATR phosphorylates Chk1 [66], which phosphorylates and stabilize p53 in a context of persisting damage and results in inhibition of cell cycle progression and induction of apoptosis [67]. This pathway is critical for the induction of senescence in conditions with persisting DNA damage such as in the DNA repair deficiency syndrome Fanconi Anemia [68-70]. The role of p53 downstream of ATR is supported by the fact that prolonged ATR activation, even in the absence of DNA damage, can promote p53-dependent senescence [71].

Activation of ATM and ATR also induces expression of the Cdnk2a locus that encodes p16 (mouse and human), p19 (mouse) and p14 (human). The mechanisms involved in activation of p53 and p16, and their role in hematopoiesis are described in part 3 of this introduction.

In this thesis we employed *Ercc1*-deficient mice to investigate how DNA repair deficiency impacts on hematopoiesis. Ercc1 is well known for its function in nucleotide excision repair (NER), but it is also involved in ICL repair at the replication fork where it functions in the Fanconi pathway [72]. Defective ICL repair requires efficient repair of double strand breaks and non-homologous end joining as a salvage pathway. The next paragraph will introduce these mechanisms.

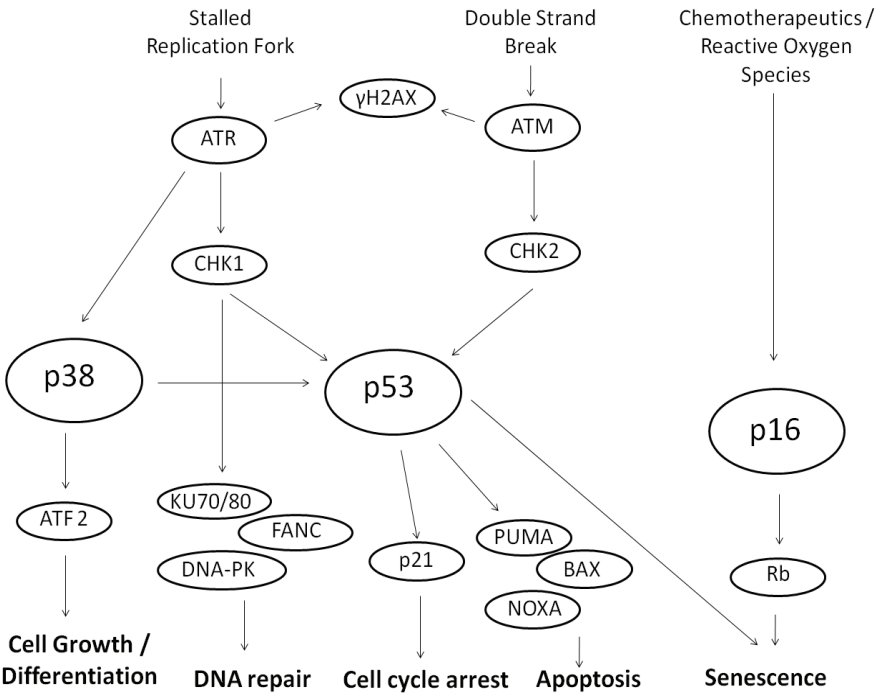

Figure 2: Major signal transduction pathways upon replication fork stalling, double strand break induction and cellular stress, focusing on cell cycle kinases and p53.

Upon stalling of replication forks ATR kinase is activated, while DSB activate ATM. In turn, ATR and ATM phosphorylate H2AX, p38 and Chk1. Via p38 and ATF2 cell growth and differentiation is regulated. Upon phosphorylation of Chk1 either DNA repair is initiated by activating Ku/DNA-PK and FANC proteins or p53 is phosphorylated which in turn can result in senescence induction, cell cycle inhibition via p21 or induction of apoptosis via PUMA/NOXA/BAX. In case of DSB induction, ATM is activated which phosphorylates H2AX and Chk2. Chk2 in turn phosphorylates p53 which can result in senescence induction, cell cycle arrest or apoptosis (as described above). Senescence can also be induced via the p16/Rb pathway. However, it is not exactly known how this pathway is activated.

2.3 Double Strand Break Repair (DSB)

Double strand breaks (DSBs) may arise from ionizing radiation (IR), X-rays, free radicals, chemicals and during replication of a single strand break (SSB) or interstrand cross links (ICLs). Of all types of DNA damage, DSBs are the most harmful since a single unrepaired DSB can cause cell death [73]. Eukaryotic cells also produce DSBs themselves. During meiosis, DSB repair is essential for accurate chromosome segregation because meiotic recombination is initiated by the formation of DSBs [74]. DSB formation is essential for producing a diverse immune system in the context of V(D)J and class-switch recombination in lymphocytes [75]. B- and T cells recognize foreign pathogens via B- and T cell receptors respectively (reviewed in [76]). By rearranging the regional genes of the lymphocyte DNA it is possible to generate a great variety in antibody repertoire.

DSB repair occurs primarily by 2 pathways: nonhomologous end joining (NHEJ) and homologous recombination (HR) (Figure 3).

NHEJ repairs DSBs by joining the 2 DSB ends together. It is a powerful pathway to repair DSBs because it can ligate all DSB ends without the requirement of a homologous sequence. By consequence, it is error-prone and may generate small insertions and deletions [77-78]. NHEJ plays a major role in DSB repair during the G1 phase of the cell cycle since HR is not efficient in this phase due to the lack of sister chromatids [79].

HR is a more error-free pathway which uses DNA homology to guide DNA repair. A DSB can be accurately repaired by using the undamaged sister chromatid strand as a template for the repair of the broken sister chromatid strand. Two intact DNA copies are yield from the ligation of the DNA strands and the separation of the joined molecules [80].

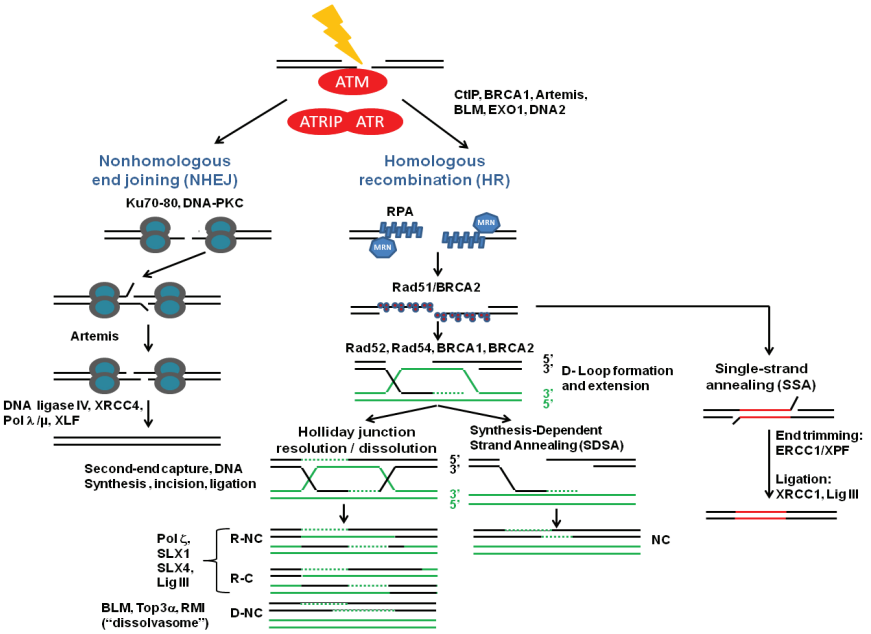

Figure 3: Mechanisms of HR and NHEJ.

The DSB signalling is initiated by ATR-ATRIP and/or ATM. In NHEJ, the Ku70/80 heterodimer binds the damaged DNA ends. This facilitates the recruitment of the DNA-PK catalytic subunit to the DSB. The sequential binding of these proteins leads to the phosphorylation of DNA-PKc and the Ku heterodimer. DSB ends may need to be processed before ligation to obtain fitting ends. Artemis removes unpairable bases after which Pol λ/μ will fill in the remaining gaps after pairing of complementary overhangs . The DNA ligase IV-XRCC4-XLF complex then ligates the juxtaposed DNA ends together. In HR the 5' DSB end resection is initiated by CtIP, Artemis and the MRN complex. Subsequently BLM complexes and EXO1/ DNA2 nucleases are recruited. End resection is completed by EXO1-BLM and BLM-DNA2 subpathways. Then RPA will bind the single strand overhangs of the DSB which will function as a substrate for Rad51 filament formation. BRCA1 recruits the BRCA2-Rad51 complex to the damaged site, after which BRCA2 loads Rad51 onto the RPA coated ssDNA.If ends are already fully compatible after resection, direct single strand annealing (SSA) will take place between complementary sequences (shown in red). The unpaired ssDNA overhangs are removed by ERCC1/XPF and the ends are ligated by DNA ligase III. If ends are not fully compatible, strand invasion will take place in the sister chromatid (homology search) and a D-loop will be formed. If elongation of the invasive strand takes place this will lead to displacement of the original homologous duplex sequence and extension of the D loop. Hereby the second DSB can be captured by annealing. This end can than also be elongated by Pol ζ (shown in dotted green) and gap filling and ligation

by DNA Polymerase III will lead to formation of a Holliday junction. Resolution of this structure by SLX1/SLX4 will lead to the formation of newly repaired DNA strands which, depending on which strand is nicked, either can lead to crossover (C) or non-crossover (NC) products. Holliday junction dissolution performed by the dissolvasome (BLM-Top3α-RMI) will specifically lead to non-crossover products. In case of Synthesis-Dependent Strand Annealing (SDSA), newly synthesized DSB ends that stick out of the D loop can be re-annealed. Sequences which are not involved in annealing are cleaved and repair can be completed by gap filling synthesis and ligation. This repair pathway always results in non-crossover products.

2.3.1 Dissease associated with defective DSB repair

Chromosomal instability is a hallmark of numerous tumor types and several diseases are linked to defective DSB repair. Deficiencies in NHEJ most often lead to an increased risk of cancer (e.g. hematological malignancies, lymphomas and ovarian cancer [81]), with enhanced chromosomal instability, including translocations which are also reported in lymphoid malignancies. Nijmegen breakage syndrome (NBS) and Bloom syndrome (BS) are inborn errors of DSB repair. Both syndromes are characterized by a high sensitivity to radiation, growth retardation and immunodeficiency, as well as by genomic instability and a high risk to develop cancer [82-83].

NBS cells lack a normal ATR-mediated response to DNA damage and fail to undergo G1/S or G2/M arrest [84]. Over 90% of all NBS cases is caused by a single hypomorphic 5 bp deletion in *Nbs1* (NBS1-657del5) which leads to two truncated fragments of nibrin; the expected 26 kD fragment (p26-nibrin) and a 70 kD protein (p70-nibrin). The nibrin protein is part of the trimeric MRN complex which consists of nibrin, Mre11 and Rad50. The MRN complex is a primary sensor for DSBs and it is required for the monomerisation and autophosphorylation of ATM [85]. The interaction between nibrin and ATM can explain the similarities which are found between A-T and NBS (see also paragraph 2.6).

BS is caused by mutations in BLM, a helicase that plays a key role in HR-dependent DNA repair [86]. It is part of the “dissolvasome” consisting of BLM, Top3α and RMI which seperates double Holiday junctions that can arise during DSB DNA repair. BLM belongs to the RecQ helicases which are involved in the separation of DNA single strands and is responsible for the maintenance of genomic stability [87]. The clinical phenotype of NBS and BS show overlap with Fanconi Anemia (see section 1.2.4), and the protein complexes that harbor the BLM or NBS1 proteins also contain FA proteins [88-89].

2.4 Nucleotide excision repair (NER)

ERCC1 is an endonuclease that functions in nucleotide excision repair (NER). It is involved in the repair of bulky and helix distorting lesions. NER is extremely versatile since it can repair various types of lesions such as UV-induced crosslinks and pyrimidine dimers [90]. The NER pathway consists of 2 separate mechanisms (Figure 4). Global genome NER (GG-NER) scans the entire genome for lesions that distort the DNA helix (e.g. bulky lesions) [91] whereas transcription coupled NER (TC-NER) only operates on lesions in actively transcribed genes [92]. The basic steps of NER are (1) damage recognition, (2) DNA unwinding and lesion verification, (3) incision of the lesion and release of the incised DNA patch, (4) repair of the created gap and (5) ligation of the newly synthesized fragment. Both pathways are described in detail in the legend to Figure 4.

2.4.1 Diseases related to defective nucleotide excision repair

At least 3 human syndromes are associated with inborn defects of NER; Xeroderma Pigmentosum (XP), Cockayne syndrome (CS), and Trichothiodystrophy (TTD). These disorders are all characterized by extreme sun sensitivity [94]. UV light induces covalent bonds between adjacent pyrimidines on the same DNA strand. The main induced lesions are cyclobutane pyrimidine dimers (CPD) and (6 – 4) pyrimidine pyrimidine photoproducts ((6 – 4)-PP).

2.4.1.1 Xeroderma Pigmentosum (XP)

XP was the first human disorder identified as a DNA repair defect [95]. It is an autosomal recessive genetic disorder in which the ability to repair UV-induced photoproducts is impaired due to mutations in XPA, XPB, XPC, XPD, XPE, XPF and XPG. The primary clinical manifestation of XP patients is a 1000-fold increase in predisposition for skin carcinoma [96]. In addition, progressive neurological degeneration is seen although this progression is highly variable between patients [97]. Clinical manifestation of this degeneration is seen as abnormal gait, sensorineural deafness and lack of deep tendon reflexes.

The increased cancer incidence can be attributed to an accumulation of mutations in the genome of actively replicating cells. In contrast, the degenerative symptoms (e.g. skin atrophy or neuron death) are probably the result from the accumulation of lesions in transcribed genes, either because they cause the inactivation of essential genes, or because they trigger apoptosis [96].

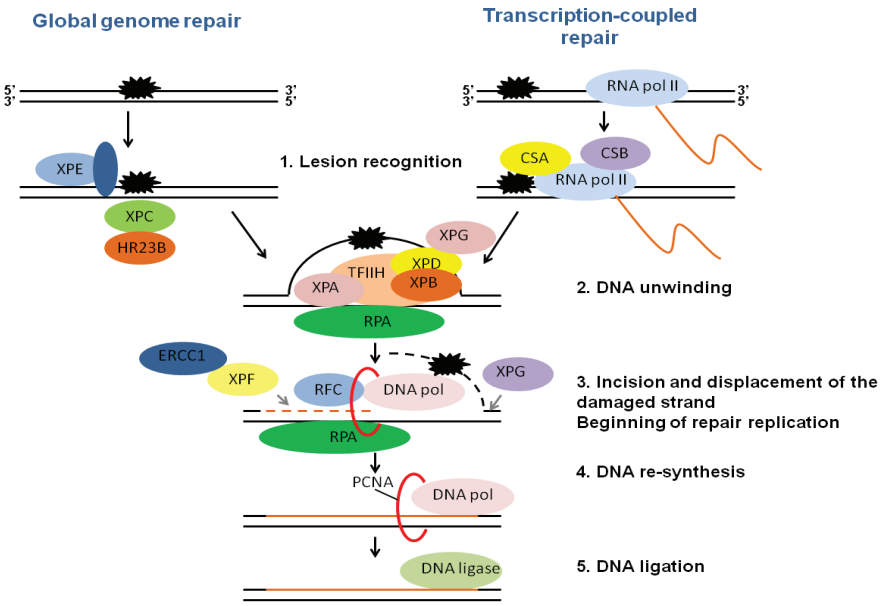

Figure 4: Mechanism of nucleotide excision repair.

This figure shows the molecular mechanism of nucleotide excision repair (NER) and its 2 subpathways, global genome NER (GG-NER) and transcription coupled NER (TC-NER). In GG-NER, XPC (complexed to HR23B) and XPE recognize the helix distorting lesion while in TC-NER stalled RNA polymerase II is the trigger for activation of CSA and CSB proteins. In both pathways the recognition step is followed by recruitment of the TFIIH complex (containing XPB and XPD) which unwinds the helix around the damaged site to form an open complex. This complex is stabilized by XPG. Next XPA verifies the lesion and RPA stabilizes the open intermediate structure by binding single-stranded DNA. XPA acts as a scaffold for binding to TFIIH, RPA and ERCC1/XPF [93]. The structure specific endonucleases XPG and ERCC1/XPF are recruited and cleave 3' and 5' of the lesion respectively, thereby releasing a 24-32 nucleotide fragment which contains the lesion. The remaining single-strand gap is filled by regular replication machinery (consisting of PCNA and DNA polymerase (recruited to the site by RFC)) and the resulting nick is sealed by ligase I or ligase III (adapted from Hoeijmakers, Nature 2001).

2.4.1.2 Cockayne syndrome (CS)

CS is an autosomal recessive disorder. It is caused by mutations in either CSA or CSB and patients are impaired in the TC-NER pathway of actively transcribed genes. CS patients exhibit extremely short stature, ataxia and progeroid appearance. The disease results in a severe reduction of lifespan but there is no link to increased cancer incidence [98].

2.4.1.3 Trichothiodystrophy (TTD)

Like XP and CS , TTD is an autosomal recessive disorder characterized by brittle hair and skin disorders [99-100]. Up to now four responsible genes have been identified for TTD; XPB, XPD, TTDA and TTDN1. TTD patients fall into three complementation groups that correspond to mutations in subunits of the general transcription factor TFIIH: TTD-A, XPB, and XPD [101]. The function of TTDN1 is still unknown. Patients often have an unusual facial appearance with protruding ears and a receding chin. Mental abilities range from the low end of the normal range to severe retardation [102]. Several categories of TTD can be recognized based on cellular responses to UV damage and the affected genes. Photosensitive cases of TTD involve mutations in XPB, XPD or TTDA. The non-photosensitive form of TTD is genetically heterogeneous with about 18% of cases relatable to a mutation in the TTDN1 gene [103]. Although patients with TTD do not exhibit increased incidences of skin cancer, mice with a human TTD mutation do show increased incidences of UV-induced skin cancer [104-105].

2.4.2 NER mouse models

For almost all NER factors mouse models have been generated [106-107] in which the NER disease phenotype is accurately preserved although cancer predisposition is more pronounced and neurological complications are milder [108]. Mice also show signs of premature aging [109]. Whether aging of the hematopoietic model occurs has only rarely been investigated. In a mouse model of TTD it has been shown that there is a progressive loss of BM progenitors while the HSC levels are preserved [110]. Analysis of the HSCs showed that they have impaired repopulation ability in a competitive BM transplantation setting compared to wildtype HSCs. This directly shows that HSC function is compromised in *Xpd*^{TTD} mice. Previously we have shown that 3 week old *Ercc1* knockout mice exhibited multilineage cytopenia and fatty replacement of bone marrow [111]. In

addition to these features we saw a reduction in stress erythropoiesis and early hematopoietic progenitors which was not seen in XPA deficient mice. These data suggest that spontaneous ICLs contribute to the functional decline of the hematopoietic system which is associated with aging.

2.5 Interstrand crosslink repair (ICL)

Interstrand crosslinks (ICLs) are very toxic to cells. Already a small number of 40 unrepaired ICLs can kill a repair deficient cell [112]. ICLs can be repaired by different DNA repair pathways, depending on how they are recognized. In the context of non replicating DNA, the distortion of the helical structure will recruit proteins involved in GG-NER. In case of RNA polymerase II stalling during transcription, TC-NER will become activated. When ICLs are detected during replication (Figure 5), it will lead to recruitment of the Fanconi Anemia pathway of ICL repair.

Stalled replication forks locally increase single strand DNA which results in activation of ATR kinase (see section 2.2), phosphorylation of Chk1 and subsequent triggering of S phase checkpoints. When ICLs result in a stalled replication fork, ATR kinase is activated, which results in association of the Fanconi core complex (FANC-A, -B, -C, -E, -F, -G, -L and -M) and its translocation to the nucleus where it associates with several other Fanconi proteins. FANC-M can also function independently of the core complex and has a role in replication fork progression and stabilisation [113]. FANC-M associates with FAAP24 and has an essential role in accumulating and phosphorylating RPA at the damaged site [114]. It is also thought that FANC-M can recognise the ICL [115].

ATR simultaneously phosphorylates FANCI, which recruits the ubiquitin ligase FANCL and leads to monoubiquitination of FANCD2 [116]. The ubiquitination of FANCD2 results in translocation of the FANCD2/FANCI complex to the nucleus where it colocalizes with several downstream FA proteins among which are FANCD1 (BRCA2), FANCN, FANCJ, FANCP and FANCO, together with DNA repair proteins among which BRCA2, Rad51, γH2AX and PCNA. Together these proteins form the so-called DNA repair foci [117]. The FANCD2/FANCI complex is involved in the incision step to remove and repair the ICL. The endonuclease FAN1 interacts with the mono-ubiquitinated form of FANCD2 and thereby is recruited to the damaged site [118]. The SLX4 protein (FANCP) coordinates the action of FAN1 as well as that of the endocnucleases MUS81/EMR1 and ERCC1/XPF. While FAN1 and/or MUS81/EME1 nicks the 5' side of dsDNA,

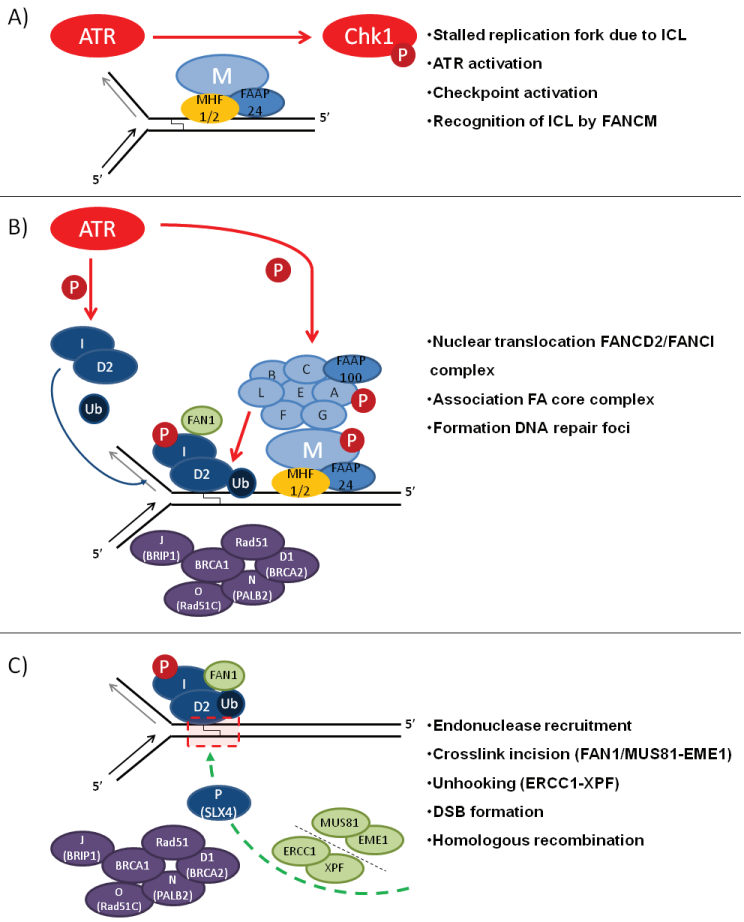

Figure 5: Schematic representation of replication coupled ICL repair.
ICLs physically block replication. When a replication fork encounters an ICL it will stall in front of it. ***A).*** *ATR will become activated and will phosphorylates Chk1, thereby inducing checkpoint activation. Simultaneously the FANCM-FAAP24 complex recognizes the ICL and FANCM is phosphorylated by ATR.* ***B)****. ATR simultaneously phosphorylates FANCI which leads to recruitment of FANCL and assembly of the FA core complex. FANCL monoubiquitinates FANCD2, which results in the nuclear translocation of the FANCD2/FANCI complex. There the endonuclease FAN1 associates with FANCD2. The ubiquitinated form of FANCD2 interacts with several downstream proteins such as FANCD1 (BRCA2), FANCJ (BRIP1), FANCN (PALB2), FANCO (Rad51c), BRCA1 and Rad51 to form DNA repair foci.* ***C)****. Endonuclease FAN1 interacts with ubiquitinated FANCD2, whereas the action of endonucleases ERCC1/XPF and MUS81/EME1 is coordinated by FANCP (SLX4). The endonucleases incise the crosslink and unhook it from the DNA strand. The resulting DSB can be repaired by HR.*

ERCC1/XPF makes an incision at the junction of dsDNA and ssDNA thereby unhooking the ICL. Due to ICL unhooking a DSB is formed which can then be repaired by HR as described in section 2.3. Interestingly, mice which lack *Slx4* have a defect in the recruitment of ERCC1/XPF onto chromatin which indicates a role for ERCC1 in FA mediated ICL repair [119-120].

Genetic evidence also indicates that the endocnulease ERCC1/XPF participates in the same ICL repair pathway as the FA proteins [121]. First, *Xpf* and *FancD2* are genes that suppress each other's effect which makes them likely to function in a similar pathway [122]. Second, it has been shown that in the absence of the endonuclease ERCC1/XPF complex, cells show a defect in FANCD2 recruitment to the damaged chromatin [121]. Thirdly, the phenotype of *Ercc1* knockout mice has significant overlap with FA features [111, 123-124].

2.6 Linking deficient DNA repair to disease phenotypes

The DNA damage response (DDR) operates in a variety of biological situations outside of its core business of DNA repair. Such as generation of immune diversity via V(D)J recombination, genetic diversity generation via sexual reproduction and regulation of telomere homeostasis. A tight regulation of the DDR is thus of critical importance for the cell to maintain its genomic integrity. Inherited DDR defects commonly predispose patients to cancer, contribute to mutations and may allow for survival benefits of tumor cells despite the presence of genomic instability (see Figure 6 for a schematic representation).

Ataxia Telangiectasia (A-T; caused by mutations in ATM [125]) and Ataxia Telangiectasia Related (Seckel syndrome, caused by mutations in ATR [126]) cover many aspects of the syndromes discussed in the previous paragraphs, because the surveillance role of the mutated proteins functions at the top of the DNA repair cascade. The central role of the MRN complex in the ATM dependent DSB response is exemplified by the human disorders caused by defects in the MRN complex that are regarded as 'A-T-like' conditions (A-T-LD [127]). In fact, NBS and A-T-LD exhibit a similar DSB repair defect to that of A-T [128]. A-T-LD is caused by hypomorphic defects in the Mre11 component of the MRN complex [129]. Clinically, A-T-LD manifests as a mild/attenuated version of A-T but without cancer predisposition [127]. It is very likely that mutations in specific DNA repair pathways will lead to specific disease phenotypes. However it is still not exactly clear which specific mutations underlie the phenotype of DNA repair related diseases.

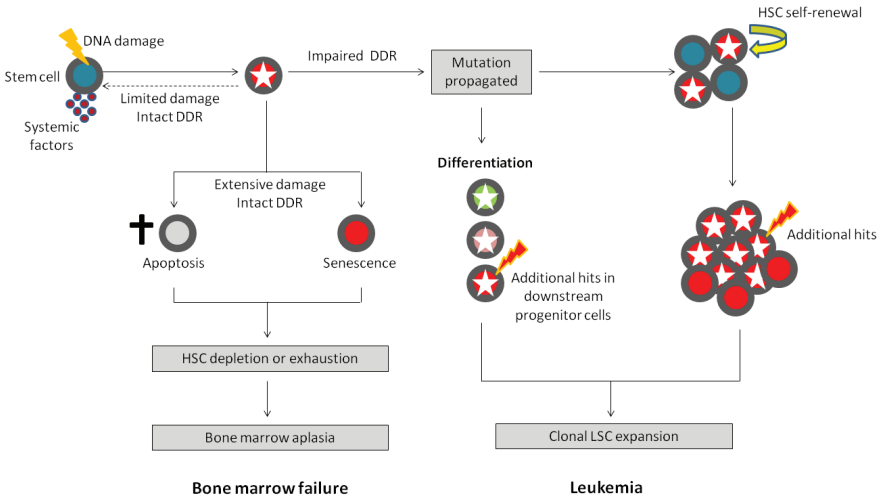

Figure 6: Cancer versus aging in the hematopoietic system.
Extensive DNA damage in the presence of an intact DDR can lead to either apoptosis or senescence depending on the type of damage. This in turn can lead to depletion of the hematopoietic system and eventually in bone marrow aplasia. Limited damage in the context of an impaired DDR can lead to mutations which can be propagated in either HSCs or progenitors. Additional hits can then lead to clonal expansion and eventually to leukemia development.

2.6.1 Cancer

Cancer predisposition is often associated with DDR deficiencies. For instance, Fanconi Anemia patients have defects in ICL repair and an increased risk of hematological malignancies. XP patients have defects in NER and are therefore hypersensitive to UV induced damage and therefore predisposed to skin cancers. A defect in mismatch repair (MMR), functioning in correcting mismatched bases generated during DNA replication, leads to predisposition to colorectal cancers in patients with hereditary non-polyposis colon cancer. Other syndromes with cancer predisposition have defects in cell cycle checkpoint activation such as AT patients, which have mutations in ATM. Besides an increased cancer risk, they also display neurodegenerative disease.

Most cancers do not show germline mutations in DNA repair genes, but they fail to cope with the mutational pressure on the cells. Tumor progression is highly correlated to a progressive impairment of DDR [130]. It is also likely that during the pre-malignant tumor state, increased oncogene-induced proliferation will lead to replicative stress. In this case replication forks will stall, resulting in DNA breaks (mostly DSBs) and constitutive activation of the DDR [131]. Unrepaired damage in genes (including those in DDR) will cause further genetic instability.

2.6.2 Premature aging

Organismal aging is generally characterized by a declining regenerative ability, increasing homeostatic imbalance and an increase in age related diseases such as cancer [132]. Three hypothesis are proposed to explain this relationship. The first suggests that longer exposure to carcinogens will ultimately lead to a higher cancer risk [133]. The second proposes that internal shifts in the organism homeostasis may favor induction of cancer or outgrowth of latent malignant cells [134]. This hypothesis includes senescence inducing mechanisms [135]. The last hypothesis is that cancer and aging are linked to the accumulation of DNA damage over time combined with increased epigenetic gene silencing [136].

Mutations in repair pathways lead to specific segmental progeroid syndromes [137-138]. For instance, mice defective in nucleotide excision repair (NER) display features commonly observed in premature aging syndromes including runted appearance, ruffled fur and shortened life span.

A consequence of insufficient DNA repair in HSCs is stem cell failure. Stem cell function can be impaired due to increased senescence or apoptosis in response to rapid tissue regeneration attempts, such as blood cell production in a hypoplastic BM. This effect is seen to variable degrees in mice defective in the Fanconi Anemia pathway, NER, MMR and NHEJ [110, 139-141].

Spontaneous and/or endogenous DNA damage limits stress-hematopoiesis by diminishing the ability of HSCs to proliferate and self-renew. HSCs are mostly quiescent and thus replication induced damage is not likely. Progenitor cells have a higher proliferation rate compared to HSCs and are therefore more prone to DNA damage induced by replication. These cells will be preferentially lost upon damage induction while the HSCs will remain and accumulate more damage. Damaged

HSCs will respond to their extensive DNA damage load by entering a senescent state or undergoing apoptosis. In either case, the BM becomes hypocellular and BM failure will be inevitable.

Genetic pathways regulating longevity respond to accumulating DNA damage by influencing the rate of DNA damage accumulation and thereby its impact on cancer and aging [142]. The cellular response to accumulation of DNA damage is thought to be upregulation of antioxidant mechanisms, activating genome maintenance pathways and suppression of the IGF-1/GH somatotrophic axis. Active metabolism produces reactive oxygen species (ROS) which can damage the DNA [143]. The conserved IGF-1 pathway modulates the speed of organismal aging by suppressing the somatotrophic growth axis and subsequent energy metabolism under stress conditions [144-145] . Thereby the formation of ROS is prevented and thus the amount of DNA damage is reduced. This will ultimately lead to a prolongation of lifespan without excessive formation of mutations and minimal loss of tissue homeostasis.

3 TUMOR SUPPRESSOR GENES AND THE DNA DAMAGE RESPONSE

Human tumor formation is promoted by mutations in 3 classes of genes: oncogenes, DNA repair genes and tumor suppressor genes (TSG). The first cancer genes found, were those who could transform cells in a dominant way, these genes are now known as oncogenes due to their ability to drive oncogenesis. The opposite class of genes is called tumor suppressor genes. These genes suppress oncogenesis by regulating cell cycle progression and thereby safeguard the fidelity of DNA replication and chromosomal segregation. In the following paragraphs several important tumor suppressors involved in leukemogenesis will be discussed in more detail.

3.1 p53

p53 is considered to be one of the major guardians of the genome. In response to DNA damage p53 induces a transient growth arrest, allowing DNA repair. In case of extensive DNA damage, p53 promotes either senescence or apoptosis [146-147]. In approximately 50% of human cancers mutations are present in the p53 gene. In the majority of the remaining 50%, p53 function is compromised due to deregulation of up- or downstream signaling pathways [148].

3.1.1 Regulation of p53

The majority of p53 protein is localized in the nucleus which is consistent with its function as a transcription factor. p53 contains several functional domains including a N-terminal transactivation domain, a proline-rich domain, a central DNA binding domain (DBD), a nuclear localization sequence (NLS), a tetramerization domain (TET), a nuclear export sequence (NES) and a C-terminal regulatory domain (REG). All domains can be post-translationally modified which ensures a rapid adaptation to changing conditions. The best known regulator of p53 is mouse double minute 2 (MDM2) which is a ubiquitin ligase [149]. MDM2-induced ubiquitination controls nuclear export and degradation of the p53 protein. Like p53, MDM2 is subject to intensive posttranslational modifications including phosphorylation, ubiquitination, acetylation and sumoylation.

Under normal circumstances, p53 turnover is maintained at a high level by the activity of MDM2, which inhibits p53's function as a transcriptional activator and targets p53 for proteasome-mediated degradation. In turn, p53 upregulates MDM2 protein levels, forming a negative autoregulatory loop. In addition, the $p14^{ARF}$ protein ($p19^{Arf}$ in mouse) stabilizes p53 by inhibiting MDM2-dependent p53 degradation, thus specifically activating the p53 pathway. Together, these proteins maintain p53 at a steady-state level, allowing the cell cycle to be controlled.

DNA damage induces phosphorylation of MDM2, which prevents it from inhibiting p53 function. Similarly, stress signals induce phosphorylation of p53, which protects p53 from MDM2-mediated targeting for degradation. By consequence, p53 accumulates. As a result of the increased p53 levels, cells will not enter the cell cycle until the DNA damage has been repaired. If repair fails, p53 will eventually trigger apoptosis [150].

Mutations in p53 can prevent *Mdm2* transcription, or binding of MDM2, resulting in protection of p53 from degradation which will lead to increased cell cycle arrest. [151]. Tumors may also show overexpression of MDM2, constitutively suppressing p53 levels and thereby preventing cell-cycle arrest and apoptosis levels by MDM2 [152]. In addition, the viral oncoproteins SV40 large T antigen and adenovirus E1B can inactivate the regulatory functions of wildtype p53 through sequestration of p53 and/or by increasing the phosphorylation of p53. As a consequence, transformed cells express high levels of functionally inactive p53 [153-154]. In contrast, the human papillomavirus E6 oncoprotein functionally inactivates p53 by strongly increasing degradation of this protein in a manner similar to MDM2 [155].

3.1.2 p53 and senescence

Cellular senescence is characterized by an irreversible cell cycle arrest [156-157]. There are 2 different forms of senescence induction. The first is known as replicative senescence and is induced by telomere shortening which is a universal mechanism for limiting proliferative potential. Due to cell divisions telomeres erode. Dysfunctional telomeres are sensed as DSBs which will induce a DNA damage response via ATM/ATR- Chk1/2- p53-p21-Cdk and induces growth arrest (as discussed in section 2.2). [158-159]. Cells that fail to senesce in response to critically short telomeres develop chromosomal aberrations, which can result in malignant transformation [160]. Senescence induced by non-telomeric signaling is called stress induced premature senescence (SIPS). In this case other types of stress such as high ROS levels [161], DNA damage [162] or abnormal oncogene activity [163] activate ATM and ATR, and elicit a senescent phenotype.

3.1.3 p53 and apoptosis

Apoptosis is a tightly regulated multistep process which regulates cell death. It is characterized by cell shrinkage, chromatin condensation as well as nuclear- and cell fragmentation. These features result in the formation of apoptotic bodies that are engulfed by neighboring phagocytic cells [164-165]. Under a variety of cell-death-inducing conditions (e.g. IR and ischemia), p53 rapidly localizes to mitochondria. Once at the mitochondrion, p53 induces mitochondrial outer membrane permeabilization (MOMP), thereby triggering the release of pro-apoptotic factors (tBid and BAX) from the mitochondrial intermembrane space [166]. Mitochondrial p53 can also release BAK from inhibitory BAK–MCL-1 complexes, thereby enabling BAK-dependent apoptotic cell death [167]. Another possibility is that the p53 target PUMA can release p53 from inhibitory p53–BCL-XL complexes [168]. Cytosolic p53 can then promote mitochondrial translocation of BAX and subsequent apoptosis [169]. p53 can also induce it's target genes PUMA and NOXA via transcriptionally regulated mechanisms [170].

3.1.4 p53 in hematopoiesis and leukemogenesis

p53 mutations in AML are associated with a complex karyotype [171], and with shorter survival and resistance to therapy [172]. The incidence of p53 mutations in AML varies from 10-15% [173-174]. The incidence with which the p53 pathway is compromised is much higher due to deregulation of upstream regulatory factors. For example, MDM2 overexpression leads to inactivation of p53 without the necessity of mutations in the p53 gene [175].

Leukemia relapse may occur because currently used therapies eliminate mostly proliferating cells that constitute the bulk of the disease, but fail to eliminate quiescent leukemic stem cells (LSCs) that can reinitiate the disease after a period of latency [176]. By understanding how p53 regulates the quiescence of HSCs during steady-state hematopoiesis, it may be possible to develop therapeutic strategies that could eliminate the largely quiescent leukemic stem cell [177].

3.2 $p16^{INK4A}$/$p19^{Arf}$ locus

The *Cdkn2A* tumor suppressor locus encodes 2 different tumor suppressor proteins namely $p16^{INK4A}$ and $p19^{Arf}$ ($p14^{ARF}$ in human). This locus is frequently mutated in human cancers [178]. $p16^{INK4A}$ and Arf

function in distinct anti-cancer pathways: $p16^{INK4A}$ regulates Rb while $p19^{Arf}$ regulates p53. These tumor suppressor proteins are expressed in response to aberrant growth or oncogenic stress and are thought to function as a brake by engaging two potent anti-proliferative pathways. Both genes are transcribed from distinct, alternate first exons but share common second and third exons that are translated in alternate reading frames.

3.2.1 $p16^{INK4A}$

The $p16^{INK4A}$ protein inhibits the activity of the cyclin D1-cyclin-dependent kinase 4 (CDK4) complex that drives cell cycle progression by phosphorylating the Rb protein. Rb phosphorylation, in turn, releases the E2F transcription factors that induce transcription of genes during the S-phase of cell cycle progression [179].

Control of E2F transcriptional activity also plays a role in myeloid differentiation. Through regulation of transcription factors, such as C/EPBα and C/EBPε, myeloid cells terminally differentiate [180-181]. In hematological malignancies, the $p16^{INK4A}$ locus is frequently hypermethylated in its promoter region which leads to transcriptional silencing of its expression [182].

3.2.2 $p19^{Arf}$/ $p14^{ARF}$

$p19^{Arf}$ physically interacts with Mdm2 [183], which prevents ubiquitination of p53. Deregulation of the ARF-MDM2-p53 pathway is a common feature in human cancer either by loss of $p14^{ARF}$/p53 or by overexpression of MDM2 [184]. In addition, $p14^{ARF}$ inhibits E2F target genes such as Cyclin A and Cyclin E [185], which also results in cell cycle arrest.

Interestingly, the tumor suppressor $p19^{Arf}$ may not only inhibit cell cycle progression, but also increase DNA repair capacity by upregulation of Xpc [186]. $p19^{Arf}$ activates Xpc expression by de-repressing the promoter through disruption of the repressor complex of E2F4. It is known that mutations in both the *Ink4a/Arf* locus and the NER pathway contribute to melanoma development [187-188]. Since the loss of the *INK4A/ARF* locus is an early event in melanoma, it could directly affect NER to predispose individuals to melanoma.

3.3 Adenomatous polyposis coli (APC)

The adenomatous polyposis coli (*APC*) gene is a key tumor suppressor gene that, when mutated in the germ line, is involved in familial adenomatous polyposis (FAP). FAP is characterized by the presence of multiple polyps in the intestine [189]. In mice, homozygous APC mutations lead to embryonic lethality [190] while conditional mutations lead to a disturbance of tissue homeostasis [191-192]. These findings suggest that APC is essential for development and homeostasis and that, when it is inactivated, facilitates tumorigenesis.

It has been shown that APC plays a role in the DNA damage response. Transcription of the APC gene can be enhanced in response to alkylating agents [193]. The transcribed APC can then interact with DNA polymerase β and flap endonuclease 1 (Fen-1), thereby modulating the BER pathway of DNA repair [194]. Taking these 2 discoveries into account, APC may play an important role in carcinogenesis by determining whether cells with DNA damage survive or undergo apoptosis [195].

APC contains binding sites for numerous proteins including microtubules, Wnt pathway components such as β-catenin and axin as well as cytoskeletal regulators. Most mutations in APC lead to a C-terminal mutation of the protein [196]. This C-terminal part of the protein contains the domains required for β-catenin and microtubule binding. β-catenin plays a role in cell adhesion via adherens junctions [197] as well as acting as a signal transducer for the Wnt signaling pathway. Binding to microtubules is involved in cytoskeletal architecture. In the following paragraphs the role of β-catenin and Wnt signaling will be discussed in more detail.

3.3.1 Wnt signaling

There are 19 known Wnt proteins which can be assigned to 2 overlapping groups with respect to their function in hematopoiesis [198]. Members of the first group, consisting of Wnt-1/2b/3/3a/4/5a/8a/8b/10b, activate signaling pathways through the canonical pathway involving β-catenin. Members of the second group, consisting of Wnt-3a/4/5a/5b/6/7/11, activate alternative non-canonical signaling pathways. The classification of Wnt ligands as either canonical or non-canonical is too simple. Recent reports show that Wnts can signal through both types of pathways, with opposing outcomes; it all depends on the cellular context [199].

3.3.1.1 Canonical Wnt signaling

The canonical Wnt signaling pathway is the best understood Wnt pathway. The proteins involved are highly conserved among species. This pathway regulates β-catenin activity and thereby controls transcription of specific target genes.

Without Wnt signaling, β-catenin is sequestered in the cytoplasm within a degradation complex which is formed by APC, axin and GSK3-β [200]. This complex phosphorylates the N-terminus of β-catenin and thereby triggers its ubiquitination and subsequent proteosomal degradation. When Wnt protein binds to the Frizzled/LRP receptor complex, Disheveled (Dsh) becomes phosphorylated and interacts with axin in order to prevent GSK3-β from phosphorylating β-catenin. This results in accumulation of β-catenin protein and its subsequent translocation to the nucleus [201]. Nuclear β-catenin regulates gene expression via TCF/LEF transcription factors [202]. Regulated genes include regulators of cellular proliferation, survival, developmental control and genes involved in tumorigenesis (The reader is referred to http://www.stanford.edu/~rnusse/pathways/targets.html for an up to date overview).

3.3.1.2 Non-canonical Wnt signaling

The most well-recognized categories of non-canonical signaling are the planar cell polarity pathway (PCP) and the Wnt calcium signaling pathway.

The calcium pathway involves activation and membrane association of phospholipase C (PLC) through GTP binding proteins [203]. PLC hydrolyses membrane phospholipids to produce di-acyl glycerol (DAG), and inositol 1,4,5-triphosphate (IP3). IP3 induces the release of calcium from the endoplasmic reticulum by association with an ATPase pump, this in turn increases the expression and activity of calmodulin and calmodulin kinases [204]. Increased intracellular calcium can activate protein kinase C (PKC), which can also be directly activated by DAG. Activation of PKC can influence a range of cellular functions including motility, apoptosis and differentiation, which in turn regulate processes such as morphogenesis. PKC can also regulate the expression of Wnt5a resulting in a positive feedback loop [205].

The PCP pathway is involved in regulation of planar cell polarity in Drosophila. In the PCP pathway, Wnt activates Rho/Rac GTPase and Jun-N-terminal sequence kinase, which modulate the organization of the cytoskeleton and influence gene expression.

In addition, Wnt factors may bind the alternative receptors Ryk or Ror. Signalling through these receptors inhibits canonical Wnt signaling by other Wnt factors.

3.3.1.3 Wnt signaling in hematopoiesis

Hematopoietic tissues express a number of Wnt family members including Wnt-2b, -3a, -5a and -10b and their receptors Fzd-3, -4, -5 and -7 [206-208]. Wnts are produced by the hematopoietic cells themselves as well as by non-hematopoietic components of the BM such as stromal cells, which produce Wnt-5a and Wnt-3 [209]. The more primitive $CD34^+$ cells also express Wnt-5a [207]. This provides the opportunity for both autocrine and paracrine stimulation of HSCs by Wnts within the BM. Wnt overexpression increases proliferation of $CD34^+$ cells and it is essential in HSC self-renewal [210].

The involvement of the Wnt pathway in regulation of hematopoietic stem and progenitor cell growth and self-renewal, in combination with its oncogenic potential in other cell types suggests that it might be deregulated in hematological malignancies. Indeed, aberrant Wnt signaling leads to oncogenic growth in both lymphoid (T-ALL, B-ALL, MM, NH-L, CLL) and myeloid malignancies (AML, CML) [211]. Results from several studies indicate that high expression of ß-catenin is a common denominator in myeloid leukemic cells. Unlike solid malignancies which display activating mutations in components of the Wnt pathway, it is more likely that in leukemias there is an autocrine stimulation of the Wnt pathway which leads to oncogenesis. On the other hand, non-canonical Wnt signaling e.g. by Wnt5a, can inhibit proliferation by limiting signaling through the canonical pathway of β-catenin–TCF activation [212].

4 EXCISION REPAIR CROSS COMPLEMENTING-GROUP 1 (ERCC1)

4.1 The ERCC1 protein

As described briefly in section 2.4, ERCC1 functions in NER. In NER, ERCC1 heterodimerizes with XPF to form an endonuclease involved in processing DNA adducts during NER, DNA replication and meiotic recombination [213]. The dimerization with XPF is crucial for the stability and catalytic activity of ERCC1 [214].

The human *ERCC1* gene is located on chromosome 19q13.2-19q13.3. It consists of 10 exons which are spread over 15 kB of the genome. The protein contains 2 functional domains; a central domain which preferably binds to 5' ss overhangs [215] and a C-terminal domain which is involved in XPF binding [216]. Recently also an XPA binding site is discovered, which is located in the central domain and is involved in the proper positioning of the ERCC1-XPF complex on the damaged DNA strand [217-218]. The N-terminal domain seems to be non essential for NER and is of unknown structure [219].

The *ERCC1* promoter is located about 170 basepairs upstream from the transcriptional start site. This region contains an AP-1 like binding site which seems to be critically involved in regulating *ERCC1* expression [220]. The DNA crosslinker cisplatin has been shown to induce a dose- and time dependent increase in *ERCC1* mRNA and protein expression in ovarian cancer cell lines which suggests that DNA repair capacity is correlated to expression [221-222]. This would imply that *ERCC1* mRNA levels could be used as a marker for DNA repair capacity in human tumors. However up to now no studies have been published which show that increased ERCC1 expression also leads to increased DNA repair.

4.2 ERCC1-XPF mutation in humans (XFE)

Although ERCC1-XPF is involved in multiple genomic maintenance pathways, patients with mutated XPF usually only show mild XP symptoms and only rarely develop skin tumors before adulthood [223-224]. In all cases mutations are hypomorphic, therefore residual protein levels are present and as a consequence functional endonuclase activity remains.

Only recently the first *ERCC1* mutation in a patient was discovered, although the screening of patients was extensive for over 30 years [225]. This patient displayed only mild impairment of NER, comparable to XPF patients. However, a severe pre- and postnatal growth deficiency was present and the patient died at the age of 14 months. Genetic screening revealed that the patient carried 2 point mutations in the *ERCC1* gene, one of which resulted in a premature stop codon. The protein encoded from this allele lacked the entire C-terminal domain which is essential for XPF binding. Therefore this allele can be expected to function as a complete null. The other allele also had a point mutation but this did not seem to influence enzymatic activity. It is very likely that the clinical phenotype of the patients is a result of the low levels of ERCC1-XPF rather than the reduced enzymatic activity.

4.3 Ercc1 mouse models

In mice, both *Ercc1* and *Xpf* knockout mutants are viable [226-227]. However they have a very severe phenotype. Embryonic and early postnatal development is mildly retarded in Ercc1 deficient mice, but in the second week of life a dramatic growth arrest develops to which the mice succumb between day 19 and 21. *Ercc1*$^{-/-}$ mice show signs of premature aging in skin, liver and BM [111, 124, 228]. In addition they display neurodegeneration, renal insufficiency, loss of muscle mass and strength, a hunchback spine and on a cellular level they suffer from premature replicative senescence and oxidative stress sensitivity [145]. Hypomorph Ercc1 (*Ercc1*$^{-/d}$) mice harbor a C-terminally truncated allele expressing an Ercc1 protein that fails to heterodimerize with Xpf. *Ercc1*$^{-/d}$ mice display a similar phenotype as *Ercc1*$^{-/-}$ but as a result of their residual DNA repair capacity they survive somewhat longer (~6 months).

Unlike all other NER models, Ercc1 mice display hematopoietic defects similar to FA deficient cells [111]. In addition they display severe organismal aging together with ICL hypersensitivity. The hematopoietic defect is related to accelerated aging of the BM causing loss of HSCs within 3 weeks after birth. Accelerated aging of the BM is also seen in mice which are defective in the FA pathway, but to a lesser extent [140]. In FA patients and mouse models there are also other reminiscent premature aging features such as growth hormone deficiency and premature gonadal failure leading to infertility [229-231].

Because Ercc1-Xpf is proposed to function in several distinct pathways of DNA repair it is necessary to be able to segregate it's function. In NER, it is Xpa that recruits Ercc1 to the site of damage by interacting with it [217, 232], while it is thought that in ICL repair this is done by Slx4 [119-120]. This is supported by the fact that Xpa deficiency leads to an increase in UV sensitivity while ICL sensitivity is not affected [145]. In contrast, loss of Slx4 leads to an increase in ICL sensitivity and does not affect UV sensitivity [233].

Although Ercc1 is not exclusively required for activation of the FA pathway (as defined by continuing presence of Fancd2 monoubiquitination in the absence of Ercc1) it does affect the kinetics of Fancd2 ubiquitination. In the absence of Ercc1, prolonged ubiquitination of Fancd2 is seen which hints that Ercc1 functions downstream of Fancd2 and that it has an essential function in incising the crosslinked DNA during ICL repair and in subsequent DNA damage response termination.

5 SCOPE OF THIS THESIS

The molecular processes that drive leukemogenesis from senescent bone marrow are largely unknown. The aim of tis thesis is to understand the molecular basis of BM senescence in response to DNA damage, and how these steps subsequently contribute to leukemogenesis.

Ercc1 deficient mice are deficient in both NER and ICL repair and display signs of premature aging in the BM. Because Ercc1 is involved in the FA pathway of ICL repair we used this model to investigate the molecular mechanisms of BM failure and subsequent leukemogenesis as seen in FA. Important questions in this respect are: **(i)** Is BM failure specific to defective ICL repair, or to the overall aging phenotype (Chapter 2), **(ii)** what is the influence of Ercc1 levels on senescence in the hematopoietic system (Chapter 3), **(iii)** which tumor suppressor mechanisms are responsible for BM failure as seen in *Ercc1* deficient mice (Chapter 4), and **(iv)** is the poor response to Epo specific to the lack of Fanconi proteins or secondary to senescence (Chapter 5). Finally, we investigated whether activation of p53 counteracts Wnt signaling (Chapter 6). In order to address these questions we analyzed *FA* deficient erythroblasts isolated from fetal liver (Chapter 5), compared the phenotypes of $Ercc1^{-/-}$, $Ercc1^{-/d}$ and $Ercc1^{d/d}$ mice (Chapter 3), generated double knockout mice which lack either the tumor suppressor gene *Cdkn2A* ($p16^{INK4A}/p19^{Arf}$) or *p53* in an *Ercc1* hypomorph background (Chapter 4) and analyzed the effect of the Wnt signaling pathway in primary erythroblasts derived from fetal livers (Chapter 6).

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2

Hematopoiesis is not affected in prematurely aged mice that lack nucleotide excision repair

Judith Verhagen-Oldenampsen [a], Jurgen R. Haanstra [a], Paulina M.H. van Strien, Marijke Valkhof, Ivo P. Touw and Marieke von Lindern

[a] These authors contributed equally

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Abstract

Background

The occurrence of bone marrow failure increases in the elderly population and constitutes a risk factor for hematopoietic malignancies. Increasing DNA damage is thought to contribute to bone marrow failure, but the relation between aging, DNA repair and bone marrow failure remained unclear. The *Ercc1*$^{-/-}$ and *Xpa*$^{-/-}$*Csb*$^{m/m}$ mouse models lack nucleotide excision repair and show a comparably severe aging phenotype due to suppression of the somatotrophic growth axis, reduced Igf-I signalling, and subsequent reduction of energy metabolism. The endonuclease Ercc1 is involved in NER, but also in interstrand cross link (ICL) repair. We previously showed that the colony forming capacity of *Ercc1*$^{-/-}$ mice is severely impaired. Here we investigate whether hematopoiesis is impaired by severe aging due to suppression of the somatotrophic growth axes for which we compare hematopoiesis between *Xpa*$^{-/-}$*,Csb*$^{m/m}$ mice that lack NER, and *Ercc1*$^{-/-}$ mice that lack both NER and ICL repair.

Results

At 16 days of age both mouse models showed a severe growth delay. Loss of Ercc1 resulted in loss of hematopoietic stem cells from bone marrow (BM) and impaired colony forming ability of progenitor cells. The severe growth defect in *Xpa*$^{-/-}$*,Csb*$^{m/m}$ mice, however, was not associated with loss of hematopoietic stem cells, or with impaired colony formation of myeloid progenitors.

Conclusions

Premature aging and growth inhibition due to increasing DNA damage that ought to be repaired by NER is not associated with loss of hematopoietic capacity. The loss of hematopoietic potential is specific for Ercc1-deficient mice, which suggests that it is restricted to defective ICL-repair.

BACKGROUND

Aging is associated with an increased occurrence of bone marrow failure which constitutes a risk factor for hematopoietic malignancies. DNA damage is thought to contribute to bone marrow failure, but the relation between aging, DNA repair and bone marrow failure remaines unclear. The cellular response to DNA damage consists of the recruitment of damage-specific DNA repair enzymes, the care-takers, and the activation of proteins that prevent the replication of damaged DNA; the gate keepers [1]. Although DNA damage can be expected to impair cell growth in general, different DNA repair deficiencies result in distinct phenotypes, which may be due to the activation of distinct gatekeepers.

We previously reported that the BM of mice lacking the endonuclease Ercc1 ages prematurely [2]. Whereas the peripheral blood of *Ercc1*$^{-/-}$ mice is still largely normal at 3 weeks of age, hematopoietic stem- and progenitor cells (HSPC) in the BM are reduced in number, and fail to proliferate *in vitro*. Ercc1 is involved in both NER and the repair of ICLs [3-4]. ICL repair is impaired in the bone marrow failure syndrome Fanconi anemia, and the ICL-repair defect of *Ercc1*$^{-/-}$ mice is a valuable model to investigate hematopoietic defects as seen in Fanconi anemia [5].

The NER pathway is required for the removal of helix-distorting lesions, which are typically induced by UV irradiation. NER is initiated by two distinct damage recognition pathways, global-genome NER operating throughout the genome and transcription coupled excision repair which is activated upon stalling of RNA polymerase II [6]. Defects in NER can lead to either highly elevated skin cancer susceptibility, as seen in Xeroderma Pigmentosum (XP) patients who carry mutations in the NER genes XP-A,B,C,D,E,F or G [7]. On the other hand patients with Cockayne syndrome (CS), that carry mutations in the transcription coupled-NER specific genes CSA or CSB, or with Trichothiodystrophy (TTD), caused by mutations in XPD as well as XPF-Ercc1, mainly show progeroid/ premature aging-like phenotypes [8]. *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice develop severe growth retardation and accelerated aging and die within three weeks postnatal, shortly before *Ercc1*$^{-/-}$ mice. Growth retardation in *Ercc1*$^{-/-}$ and *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice appeared to be due to suppression of the somatotrophic growth axis, reduced Igf-I signalling, and subsequent reduction of energy metabolism [9-10]. To distinguish the cause of progeroid features, growth retardation and bone marrow failure we investigated hematopoietic parameters in NER defective *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice and compared it to the previously studied *Ercc1*$^{-/-}$ mice. This revealed that premature aging and growth retardation associated with defective NER does not affect hematopoiesis.

RESULTS AND DISCUSSION

Similar to what was shown previously, *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice were severely growth retarded at 16 days of age, as exemplified by a 42% reduction in body weight compared to *wt* littermates [11]. Age matched *Ercc1*$^{-/-}$ mice showed a 57% reduction in body weight compared to *wt* littermate controls (Fig. 1A) [12]. Due to premature aging all *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice died before they were 19 days old, while *Ercc1*$^{-/-}$ mice died after postnatal day 21 (data not shown). This indicates that both models are very similar in their growth retardation with a slightly worse life expectancy for the *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice.

Next, we analysed the HSPC compartment. We could compare the frequency of hematopoietic subpopulations because the ratios mononuclear cells/animal weight were constant for all genotypes (data not shown). The fraction of LSK (lineage-marker negative (lin)Sca1^{+}ckit^{+}) cells, representing the HSPC compartment, in the BM of *Ercc1*$^{-/-}$ mice was reduced to 38% compared to *wt* littermates. However, there was no significant difference in the LSK frequency between *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice and their *wt* littermates (Fig. 1B).

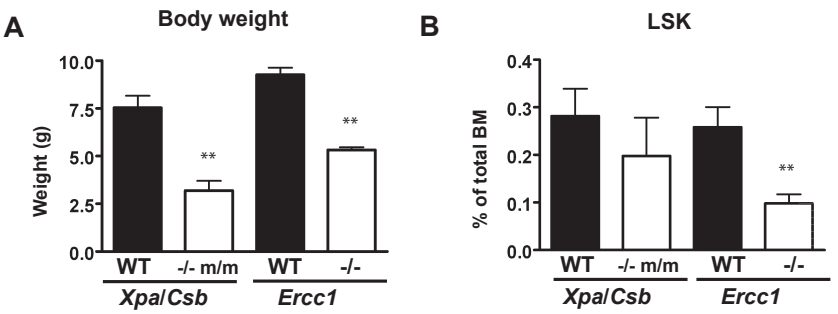

Figure 1: The effect of Xpa/Csb-deficiency and Ercc1-deficiency on mouse weight and hematopoietic stem cells

*(A) Weight of 16 day old mice in grams, and (B) Frequency of LSK cells representing the HSPC compartment. Data from 1x10^{6} BM cells were recorded. Genotypes are shown below the x-axes. Bars represent the average of at least 3 mice, error bars represent standard deviation. Two-tailed student t-tests were performed to determine significance *: p<0.05, **: p<0.001*

Progenitor frequencies were determined by immunophenotyping of common myeloid progenitors (CMP; lin^{-}ckit^{+}CD16/32^{int}CD34^{+}), granulocyte-monocyte progenitors (GMP; lin^{-}ckit^{+}CD16/32^{hi}CD34^{+}) and MEPs (lin^{-}ckit^{+}CD16/32^{int}CD34^{low}) (Fig 2A-C). This indicated that progenitor populations were similar between *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice and *wt* controls, while

they were decreased in *Ercc1*$^{-/-}$ mice compared to *wt* littermates although with a probability to be similar of 0.057 for CMP, 0.12 for GMP and 0.10 for MEP (n≥3). The capacity of *Xpa*$^{-/-}$*Csb*$^{m/m}$ bone marrow progenitors to form colonies *in vitro* was reduced to 30% in presence of G-CSF, but unaffected in presence of GM-CSF or Epo/SCF (Fig 2D-F). *Ercc1*$^{-/-}$BM, however, failed to produce any colonies in presence of G-CSF or Epo/SCF, while colony formation was reduced to 66% in the presence of GM-CSF (Fig 2D-F).

In summary, *Xpa*$^{-/-}$*Csb*$^{m/m}$ and *Ercc1*$^{-/-}$ mice were both severely growth retarded, but only Ercc1-deficient animals displayed a hematopoietic defect. These results indicate that growth retardation in *Ercc1*$^{-/-}$ mice is linked to NER-deficiency and suggest that the hematopoietic phenotype is associated with deficient ICL repair.

Using *Xpa*$^{-/-}$ mice, we previously showed that defective NER does not reduce HSPCs in the BM [2]. However, *Xpa*$^{-/-}$ mice are hypersensitive to UV-induced carcinogenesis mirroring the skin cancer susceptibility of XP patients, but do not develop the growth retardation and progeroid phenotypes that are manifested in *Ercc1* mutants or in *Xpa*$^{-/-}$*Csb*$^{m/m}$ double mutants. The data presented here point to a context-dependent sensitivity to different types of DNA damage. Loss of TC-NER as it occurs in *Csb*-deficient mice suppresses the growth hormone (GH) and insulin-like growth factor 1 (Igf-I) axis [9], which causes the severe growth retardation in *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice. However, hematopoiesis in *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice is hardly affected, and therefore we conclude that maintenance of HSPCs is independent of GH and Igf-I. The sensitivity of the HSPC compartment to defective ICL repair is supported by findings in *Brca2*$^{\Delta 27/\Delta 27}$ mice that carry hypomorphic alleles of *Brca2*, also known as *Fancd1*. Brca2/Fancd1 is required for homologous recombination downstream of the FA pathway but has no direct link to NER [13-14]. *Brca2*$^{\Delta 27/\Delta 27}$ mice have a minor growth defect (body weight is 85% of *wt*) but display a prominent hematopoietic phenotype: the colony forming capacity of *Brca2*$^{\Delta 27/\Delta 27}$ BM is 2- to 4-fold reduced, and HSPCs are outcompeted by *wt* cells in transplantation experiments [15]. In contrast to *Ercc1*$^{-/-}$ mice the *Brca2*$^{\Delta 27/\Delta 27}$ mice did not show reduced frequencies of HSPCs in the BM. This difference between the two mouse models may be attributed to different steps in ICL-repair that are affected by either mutation. However, we cannot rule out some synergistic effects of defective ICL and NER in the hematopoietic phenotype of our Ercc1-deficient mice.

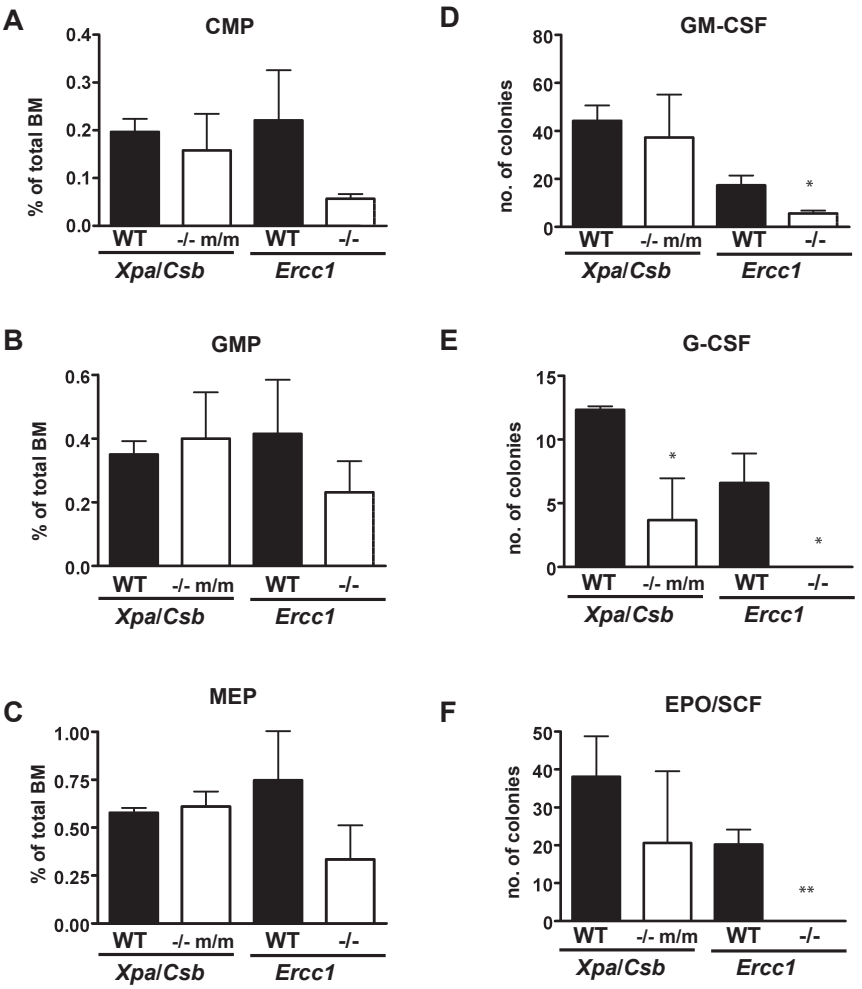

Figure 2: Progenitor frequencies and their colony forming capacity in Xpa/Csb- and Ercc1-deficient bone marrow

*(A-C) Progenitor frequencies were determined using BM cell suspensions: common myeloid progenitors (CMP; lin$^-$ckit$^+$CD16/32^{int}CD34$^+$), granulocyte-monocyte progenitors (GMP; lin$^-$ckit$^+$CD16/32^{hi}CD34$^+$) and MEPs (lin$^-$ckit$^+$CD16/32^{int}CD34^{low}). (D-F): BM cell suspensions were plated at a density of 50.000 cells per ml per dish in methyl cellulose medium containing huG-CSF (0.1 μg/ml), muGM-CSF (0.1 μg/ml), or Epo (4 mU/ml) plus transferrin (0.3 mM), hemin (0.2 mM) and muSCF (0.1 g/ml). Colonies containing 30 cells or more were scored after 7-8 days of culture. Bars show the mean of n≥3 animals. Error bars show standard deviations. Two-tailed student t-tests were done: *: p<0.05, **: p<0.001*

CONCLUSIONS

Aging is associated with an increased occurrence of bone marrow failure but the mechanism is unclear. It was previously shown that premature aging in NER-defective mice is due to suppression of the somatotrophic growth axis, reduced Igf-I signalling, and subsequent reduction of energy metabolism, a process that also occurs in mice that age naturally [9-10]. Here we show that the hematopoietic compartment of prematurely aging, NER-deficient mice is similar to that of their healthy littermates. Age-matched mice that also lack ICL repair, however, do show a significant loss of HSPC. Thus, bone marrow failure is not related to the hormonal aging physiology, but to specific types of DNA damage.

MATERIALS AND METHODS

Animals

Ercc1$^{+/-}$ [12], *Csb*$^{m/+}$ [16], *Xpa*$^{+/-}$ [17], and *wt* littermates were kept in a background of C57/Bl6 or FVB/n at the Animal Resource Center (Erasmus MC). Ercc1 experimental animals were generated as F1 in a mixed background of C57/Bl6 and FVB/n, Xpa/Csb mice were generated as C57/bl6. *Ercc1*$^{+/-}$ mice displayed a wild type phenotype and were used as controls. All animal studies were approved by an independent Animal Ethical Committee.

Mice were sacrificed by CO_2 inhalation. BM cell suspensions were obtained by crushing femurs, tibia and sternum in HBSS supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin. BM, spleen and thymus were passed through a 70 µM filter (BD Falcon, Bedford, MA) to obtain single-cell suspensions.

Colony forming assays

BM cell suspensions were plated at a density of 50.000 cells per ml per dish in triplicate in methyl cellulose medium (Methocult M3234, StemCell Technologies SARL, Grenoble, France) containing huGCSF (0.1 µg/ml), muGM-CSF (0.1 µg/ml), or Epo (4 mU/ml) plus transferrin (0.3 mM), hemin (0.2 mM) and muSCF (0.1 µg/ml). Colonies containing 30 cells or more were scored after 7-8 days of culture.

Flow cytometry

Blood and single cell suspensions from BM, spleen and thymus were analyzed by flow cytometry using a BD™ LSR II Flow Cytometer System with FCS Express Diva software (BD Biosciences., San Jose, California), and analyzed by FlowJo (Tree Star, Inc., Ashland, Oregon). Antibodies directed against CD3e, CD11b, CD45R/B220, Ly6G and Ly-6C, and TER-119 were used together with streptavidin-APC and streptavidin-Cy7 (BD Pharmingen). Directly labelled antibodies used included CD117-APC, CD16/32-PE (BD Pharmingen), Sca1-PE-cy7, CD34-pacific blue, CD127-pacific blue (ebioscience). 7-AAD (Invitrogen, Breda, the Netherlands) selected life cells. Subpopulation were gated as described by Wolfler *et al* [18]: LSK (lineage marker negative, Sca1+, ckit+), long-term hematopoietic stem cells (LT-HSC): LSK,CD34-Flt3-, short-term (ST) HSC: LSK, CD34+Flt3-, multipotent progenitors (MPP): LSK,CD34+Flt3+ common myeloid progenitor (CMP): lin-ckit+CD34+CD16/CD32low, granulocyte-monocyte progenitor (GMP): lin-ckit+CD34+CD16/CD32high and megakaryocyte-erythroid progenitor (MEP): lin-ckit+ CD34-CD16/CD32low. Data from $1x10^6$ BM cells were recorded to ensure reliable analysis of small populations.

ACKNOWLEDGEMENTS

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3

Loss of Ercc1 results in a time- and dose-dependent reduction of proliferating early hematopoietic progenitors

Judith Verhagen-Oldenampsen[a], Jurgen R. Haanstra[a], Paulina M.H. van Strien, Marijke Valkhof, Ivo P. Touw and Marieke von Lindern

[a]These authors contributed equally

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Abstract

The endonuclease complex Ercc1/Xpf is involved in interstrand crosslink repair and functions downstream of the Fanconi pathway. Loss of Ercc1 causes hematopoietic defects similar to those seen in Fanconi Anemia. *Ercc1*$^{-/-}$ mice die 3-4 weeks after birth, which prevents long-term follow up of the hematopoietic compartment. We used alternative Ercc1 mouse models to examine the effect of low or absent Ercc1 activity on hematopoiesis. Tie2-Cre-driven deletion of a floxed *Ercc1* allele was efficient (>80%) in fetal liver hematopoietic cells. Hematopoietic stem- and progenitor cells (HSPC) with a deleted allele were maintained in mice up to 1 year of age in the context of a *wt* allele, but were progressively outcompeted when the deleted allele was combined with a knock out allele. Mice with a minimal Ercc1 activity expressed by 1 or 2 hypomorphic *Ercc1* alleles have an extended life expectancy, which allows analysis of HSPC at 10 and 20 weeks of age. The HSPC compartment was affected in all Ercc1 deficient models. Actively proliferating multipotent progenitors were most affected as were myeloid and erythroid clonogenic progenitors. In conclusion, lack of Ercc1 results in a severe competitive disadvantage of HSPC and is most deleterious in proliferating multipotent progenitor cells.

1. INTRODUCTION

The Ercc1/Xpf complex is an endonuclease involved in nucleotide excision repair (NER) and in repair of interstrand crosslinks (ICL) [1 - 2]. Mice lacking Ercc1 (*Ercc1*$^{-/-}$) suffer from severe premature aging, which shows as small size, ruffled fur, liver polyploidy and loss of hematopoietic progenitors from bone marrow (BM), and results in death at 3-4 weeks of age [3-6]. Hypomorphic *Ercc1* (*Ercc1*$^{d/d}$ or Ercc1^{*292}) mice that harbor 2 C-terminally truncated alleles are also small but they survive longer (~6 months), probably as a result of their residual DNA repair capacity (~4%) [1 - 2]. The hypomorphic allele has a 7 amino acid deletion at the C-terminus which impairs dimerization with Xpf [1].

The short life span and severe aging phenotype of *Ercc1*$^{-/-}$ is shared with other models of defective NER such as the *Xpa*$^{-/-}$*Csb*$^{m/m}$ mice that die at 3 weeks of age [7-9]. The hematopoietic defect of *Ercc1*$^{-/-}$ mice, however, is specifically linked to defective ICL repair ([5]). The correlation of specific phenotypes with either NER or ICL repair is likely due to the activation of distinct tumor suppressor mechanisms that impact differently on specific tissues. For instance, persistent DNA damage due to defective NER results in deregulation of the growth axis, and is independent of p53 and p16^{INK4a} [8]. Hematopoiesis, on the other hand, is particularly sensitive to activation of p53 (Haanstra, Verhagen-Oldenampsen in preparation), chapter 4.

Both fibroblasts and hematopoietic cells of *Ercc1*$^{-/-}$ mice and mice lacking Fanconi proteins are hypersensitive to the DNA crosslinker mitomycin C (MMC) [1, 5, 10]. Importantly, the endonuclease complex Ercc1/Xpf participates in the same ICL repair pathway as the Fanconi Anemia (FA) proteins [11]. It associates with FancP/Slx4 and is required for FancD2 focus formation [12]. Mice lacking for instance the Fancc gene only develop hematopoietic defects when challenged with MMC, or when hematopoietic cells are cultured at atmospheric oxygen prior to transplantation [10, 13]. Mice lacking Ercc1 develop hypoplasia of the BM compartment without applying an external challenge similar to FA patients [14] and Fancp/Slx4-deficient mice [15].

The Ercc1 mice are a useful model to study BM failure in FA, which is, however, limited by the short life span of *Ercc1*$^{-/-}$ mice. The BM of *Ercc1*$^{-/-}$ mice contains fewer progenitors, and the remaining myeloid and erythroid progenitors fail to proliferate *in vitro* [5]. The aim of this study was to characterize progression of BM failure in Ercc1 models with an extended life span, and to examine how low levels of Ercc1 activity

impact on hematopoiesis. We used mice with a single floxed *Ercc1* allele crossed in to a Tie2-driven Cre recombinase. Tie2 is expressed in the early hematopoietic stem cell (HSC) when they dissociate from the hemogenic endothelium, and in quiescent adult HSC [16-17]. We show that the *Ercc1* allele recombines efficiently in fetal liver. In presence of an intact *Ercc1* allele the recombination frequency remained stable, while the frequency of cells lacking Ercc1 rapidly decreased in BM when the second *Ercc1* allele was lacking. This indicated that Ercc1-deficient hematopoietic cells have a severe competitive disadvantage. To investigate how low levels of Ercc1 affect hematopoietic stem- and progenitor cells we compared hematopoiesis in $Ercc1^{wt}$, $Ercc1^{d/d}$, and $Ercc1^{-/d}$ mice at 3, 10 and 20 weeks of age. At week 3 we included $Ercc1^{-/-}$ in this comparison. This analysis showed that proliferating stem- and progenitor cells decreased, whereas the most immature cells within the LSK fraction became less affected when these cells became quiescent after week 3 of age. The decrease of multipotent progenitors preceded the decrease of committed progenitors indicating that the earliest proliferating progenitors are most sensitive to defective ICL repair.

2. MATERIALS AND METHODS

2.1 Animals

$Ercc1^{+/d}$, $Ercc1^{+/-}$ [1], $Ercc1^{+/f}$ (obtained from Dr. L. Niedernhofer, University of Pittsburgh School of Medicine, Pittsburgh, PA), *Tie2-Cre* [18], and *wt* littermates were kept in a pure background of C57/Bl6 and FVB/n at the Animal Resource Center (Erasmus MC). Experimental animals were generated as F1 in a mixed background of C57/Bl6 and FVB/n. $Ercc1^{+/-}$ and $Ercc1^{+/d}$ mice displayed a wild type phenotype and were used as controls. All animal studies were approved by an independent Animal Ethical Committee. Mice were sacrificed by CO2 inhalation between postnatal week 3 and 20. Neonatal mice and embryo's were sacrificed by decapitation on ice. Femurs, tibia and sternum were isolated and BM cell suspensions were obtained by crushing the bones in HBSS supplemented with 5% (v/v) foetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Fetal livers and neonatal spleens were resuspended by pipetting in the same medium.

2.2 Colony forming unit assays

Bone marrow cell suspensions were plated in methyl cellulose medium (Methocult M3234, StemCell Technologies SARL, Grenoble, France) containing huGCSF (0.1 mg/ml), muGM-CSF (0.1 mg/ml), or Epo (4 mU/ml) plus transferrin (0.3 mM), hemin (0.2 mM) and muSCF (0.1 mg/ml). Colonies containing 30 cells or more were scored after 7-8 days of culture.

2.3 Flow cytometry

Single bone marrow cell suspensions were analyzed by flow cytometry using a BD™ LSR II Flow Cytometer System with FCS Express Diva software (BD Biosciences., San Jose, California). FCS files were analyzed using FlowJo (Tree Star, Inc., Ashland, Oregon). Cell were labelled with the following antibodies; mouse biotinylated lineage depletion kit, CD16/CD32-PE, CD117-APC, CD135-PE and streptavidin-APC-Cy7 (BD Pharmingen), Sca1-PE-Cy7, CD34-pacific blue and CD127-pacific blue (ebioscience) and 7'AAD (Invitrogen).

2.4 Genotyping PCR and Q-PCR

Genomic DNA was isolated from tail segments, or from blood (NucleoSpin Tissue XS, MACHEREY-NAGEL GmbH & Co). Genotypes were determined by PCR. Genomic Q-PCR used an Applied Biosystems 7900 instrument (Applied Biosystems, Weiterstadt, Germany) and SYBR Green PCR Master Mix (Applied Biosystems). Primers used were: HPRT - forward: AGCCTAAGATGAGCGCAAGT, reverse: ATGGCCACAGGACTAGAACA; Recombined *Ercc1* allele - forward: TGCAGCATGCTCTAGACTCG, reverse: CCATGAATTCCGGGATCTCTCGAC; Non-recombined *Ercc1* allele - forward: TCCACTTCGCATATTAAGGTGA, reverse: AACCTGCGTGCAATCCAT; *Ercc1* knock out locus - forward: TCCTCGTGCTTTACGGTATC, reverse: CAGGATCAGGAGGTACAGGA;

2.5 Histology

Livers were embedded in Tissue-Tek O.C.T (Sakura Finetek, Zoeterwoude, Netherlands). 4 µm sections were made using a cryostat (Leica) and stained with hematoxylin and eosin. Slides were imaged on a Leica DMLB light microscope equipped with Leica application suite 2.7.1 (Leica Microsystems (Switzerland).

3. RESULTS

3.1 Ercc1-deficient hematopoietic stem and progenitor cells have a competitive disadvantage

Ercc1$^{-/-}$ mice have an average lifespan of 3 weeks. Because we aimed to study long-term effects of Ercc1-deficiency on hematopoietic stem cell function, we used a Cre-lox conditional mouse model expressing Cre-recombinase from the Tie2 promoter (*Tie2-Cre*). Tie2 is expressed on vascular endothelial cells and HSCs [16-17]. Mice with a single floxed *Ercc1* allele (*Ercc1*$^{+/f}$) were crossed with *Ercc1*$^{+/-}$ *Tie2-Cre* mice. We compared *Ercc1*$^{-/f}$ and *Ercc1*$^{+/f}$ mice with and without expression of Tie2-Cre. Because the recombination efficiency in Cre-lox mouse models is never 100% [19], deletion of the floxed allele was analyzed both pre- and postnatal in the most active hematopoietic organ, *i.e.* fetal liver in the embryo, spleen in newborn animals and BM in adult animals.

The presence of the floxed allele was analyzed by real time genomic PCR on DNA isolated from the various tissues. The fraction of cells with a deleted floxed allele was calculated by comparing the relative signals in tissues with or without Cre. *Tie2-Cre/Ercc1*$^{+/f}$ mice showed stable deletion of the floxed allele in 50% or more of the hematopoietic cells (Figure 1). In *Tie2-Cre/Ercc1*$^{-/f}$ mice the Ercc1 allele was deleted in 80% of fetal liver cells at prenatal days E12.5 and E15.5. In newborn *Tie2-Cre/Ercc1*$^{-/f}$ animals (postnatal day 1) ~ 50% of spleen cells carried a deleted floxed allele. At ten weeks of age the recombined allele was undetectable or present in a low percentage of cells. In *Tie2-Cre/Ercc1*$^{-/f}$ animals of 1 year old the BM contained hardly any cells with a recombined allele (Figure 1). Accordingly, blood cell parameters and colony forming progenitors in BM were similar in *Ercc1*$^{-/f}$ mice with or without *Tie2-Cre* expression at 10 weeks and 1 year of age (data not shown). This indicates that *Ercc1*-deleted cells are outcompeted by cells in which the floxed allele was not recombined. The presence of one *Ercc1* allele is sufficient to maintain the hematopoietic cell compartment at a similar level as in nondeleted animals.

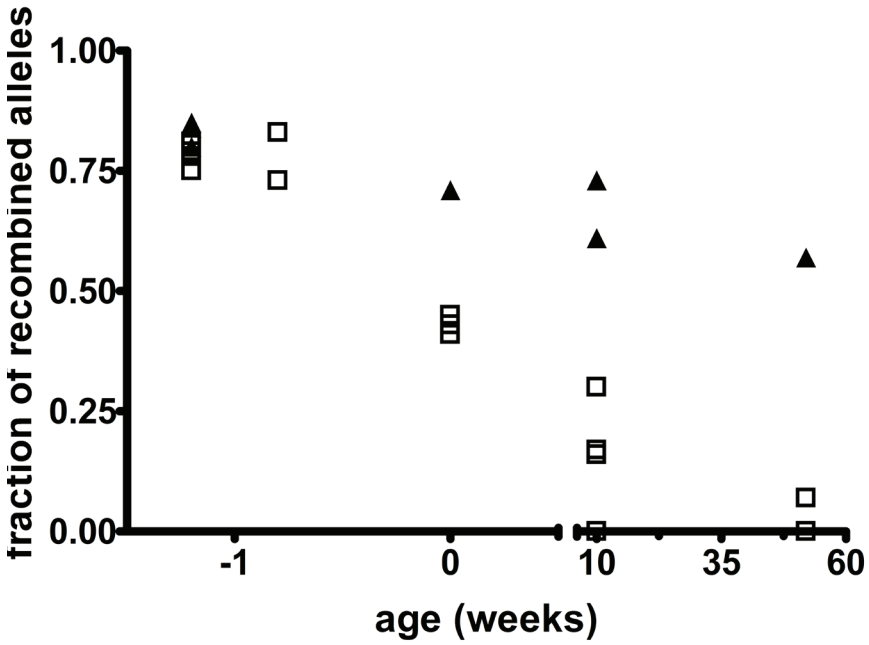

Figure 1: Recombination in Ercc1-flox Tie2Cre model.

The fraction of recombined alleles in the presence of Tie2-Cre was calculated after measuring the non-deleted floxed allele by real time genomic PCR and comparing it to the presence of the floxed allele in absence of Cre. DNA was isolated from fetal livers at embryonic day E12.5 and E15.5, from the spleen of neonatal mice, and from bone marrow of 10 and 52 week old mice. Closed triangles: $Ercc1^{+/f}$, open boxes: $Ercc1^{-/f}$. Each symbol is an independent measurement.

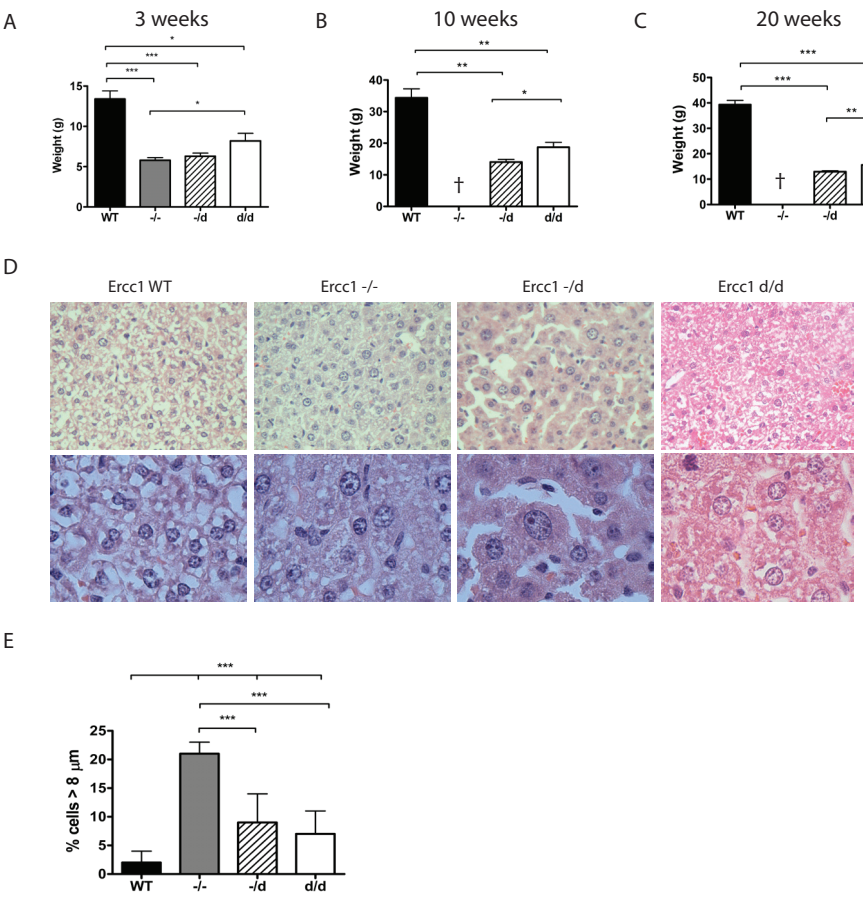

Figure 2: Weight and liver cell morphology of mice with distinct levels of Ercc1 activity.

*(A-C) Mean body weight of (A) 3 week old Ercc1$^{-/-}$ (n=6), Ercc1$^{-/d}$ (n=7), Ercc1$^{d/d}$ (n=4) and wt (n=13) mice. (B) 10 week old Ercc1$^{d/d}$ (n=3), Ercc1$^{-/d}$ (n=3) and wt (n=6) mice. (C) 20 week old Ercc1$^{-/d}$ (n=8), Ercc1$^{d/d}$ (n=5) and wt (n=12) mice. (D) Hematoxylin- and eosin-stained sections of liver from 3 week old wt, Ercc1$^{-/-}$, Ercc1$^{-/d}$ and Ercc1$^{d/d}$ mice. (E) Quantification of enlarged nuclei (>8µm). Error bars indicate standard deviation. * indicates p≤0.05, ** indicates p≤ 0.01 and *** indicates p≤0.001.*

3.2 The composition of the hematopoietic stem cell pool is affected by the level of Ercc1 activity

To find a window of Ercc1 expression that allows for the analysis of hematopoiesis for several weeks, we compared hematopoiesis in bone marrow of *Ercc1*$^{-/-}$ mice with mice harboring one C-terminally truncated *Ercc1* allele and a knock out allele (*Ercc1*$^{-/d}$), or two C-terminally truncated *Ercc1* alleles (*Ercc1*$^{d/d}$). The truncated allele has been described as **293* [1] or as *delta* [20], we adopted *delta*, indicated as '*d*' that should not to be confused with a recombined floxed allele. Three week old mice with low or absent Ercc1 activity had a dose-dependent decrease in body size (Figure 2A). *Ercc1*$^{-/-}$ mice died between week 3 and 4. The *Ercc1*$^{d/d}$ and *Ercc1*$^{-/d}$ mice survived longer, but their low body weight persisted at 10 and 20 weeks of age (Figure 2B,C). A comparison of liver morphology of the various Ercc1-deficient mice at 3 weeks of age indicated that livers from both *Ercc1*$^{-/-}$, *Ercc1*$^{-/d}$, and *Ercc1*$^{d/d}$ mice contained cells with enlarged nuclei, compared to *wt* livers (larger than 8 µm; Figure 2D,E) as previously described [20].

To analyze the effect of low levels of Ercc1 on hematopoiesis, we first examined the stem- and progenitor cell compartment using flow cytometry. Hematopoietic stem cells and progenitor cells (HSPCs) were defined as negative for lineage markers (Lin-) and positive for the surface markers Sca (Sca1+) and the SCF receptor cKit (cKit+), indicated as the LSK fraction. The stem cell compartment was further subdivided into long term HSC (LT-HSC, CD34-, CD-135), short term HSC (ST-HSC, CD34+ CD135-) and multipotent progenitors (MPP, CD34+ CD135+) [21].

Because BM cellularity corrected for body weight was comparable between the different genotypes at 3, 10 and 20 weeks of age, a comparison of the subset ratios was permitted between Ercc1-deficient mice and their *wt* littermates. The percentage of LSK cells in the total bone marrow of 3 week old mice was decreased to 17% of wt for *Ercc1*$^{-/-}$, 28% of wt for *Ercc1*$^{-/d}$ and 27% of wt in *Ercc1*$^{d/d}$ mice (Figure 3A). At 10 weeks of age, the percentage of LSK cells present in the BM further decreased in *Ercc1*$^{-/d}$ mice to 10% of *wt*, but stabilized to 50% of wt for *Ercc1*$^{d/d}$ mice (Figure 3B). At 20 weeks the percentage of LSK was 26% of wt for *Ercc1*$^{-/d}$ and 31% of wt for *Ercc1*$^{d/d}$ (Figure 3C). Thus, the size of the stem cell compartment correlates with Ercc1 activity, but fluctuates over time.

We next investigated how distinct subpopulations within the LSK compartment depend on Ercc1 protein activity. The distribution of LT-HSC, ST-HSC and MPP displayed relatively minor changes at week 3 (Figure 3D).

At 10 weeks of age, the fraction of actively dividing MPP was more than 3-fold decreased in both hypomorphic models (Figure 3E). The *Ercc1*$^{-/d}$ BM contained predominantly quiescent LT-HSC, while proliferating ST-HSC were the most abundant fraction in *Ercc1*$^{d/d}$ BM (Figure 3E). The enrichment of quiescent LT-HSC is in accordance with the further reduction of LSK in *Ercc1*$^{-/d}$ BM. In contrast, the LSK fraction in *Ercc1*$^{d/d}$ BM partly recovered at week 10, which is in accordance to the increase in ST-HSC fraction. At 20 weeks of age the distribution of quiescent and dividing subfractions within the population of LSK cells remained similar to the distribution at 10 weeks for both *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ mice (Figure 3F).

To specify the distribution of progenitors that arise from the LSK fraction in relation to the remaining Ercc1 activity, we analyzed the following lineage committed progenitor subsets: Common Myeloid progenitors (CMP, Lin-Ckit+CD34+CD16/CD32int), Granulocyte-Monocyte progenitors (GMP, Lin- Ckit+CD34+CD16/CD32hi), Megakaryocyte-Erythroid progenitors (MEP, Lin- Ckit+CD34-CD16/CD32low) and Common Lymphoid progenitors (CLP, Lin-CD127+Sca1/Ckit_int). At 3 weeks of age the CMP fraction of *Ercc1*$^{-/-}$ mice decreased to 46% of *wt*, the GMP fraction to 16% of *wt*, the MEP fraction to 45% of *wt* and the CLP fraction to 48% of *wt* levels (Figure 3G). In *Ercc1*$^{-/d}$ mice the progenitor subsets decreased to respectively 39%, 54%, 88% of *wt* levels and no change in CLP levels (Figure 3G). For *Ercc1*$^{d/d}$ mice these percentages were 23%, 38% and 41% of *wt* levels and no difference in CLP levels (Figure 3G). For all myeloid subsets, except the CMP compartment, the numbers increased in *Ercc1*$^{-/d}$ mice as compared to *Ercc1*$^{-/-}$ mice.

At 10 weeks of age, BM of *Ercc1*$^{-/d}$ mice contained 20% of *wt* CMP levels, 29% of *wt* GMP levels, 49% of *wt* MEP levels and 87% of *wt* CLP levels (Figure 3H). In *Ercc1*$^{d/d}$ mice these subsets contained 32%, 27%, 39% and 74% of *wt* levels respectively (Figure 3H). At 20 weeks of age *Ercc1*$^{-/d}$ BM contained 35% of *wt* CMP levels, 77% of *wt* GMP levels, 53% of *wt* MEP levels and 67% of *wt* CLP levels (Figure 3I). In *Ercc1*$^{d/d}$ mice these subsets contained 13%, 23%, 31% and 69% of wt levels respectively (Figure 3I).

In conclusion, decreased Ercc1 levels reduce all compartments of actively proliferating stem- and progenitor cells except for the CLP fraction that is only moderately affected. Despite reduced numbers of progenitors in BM, we observed normal cell numbers in peripheral blood (data not shown). The presence of a hypomorphic *Ercc1* allele extends the life span of the mice, and marginally improves hematopoiesis in the mice. Also in *Ercc1*$^{d/d}$ mice the number of HSPC remains severely compromised.

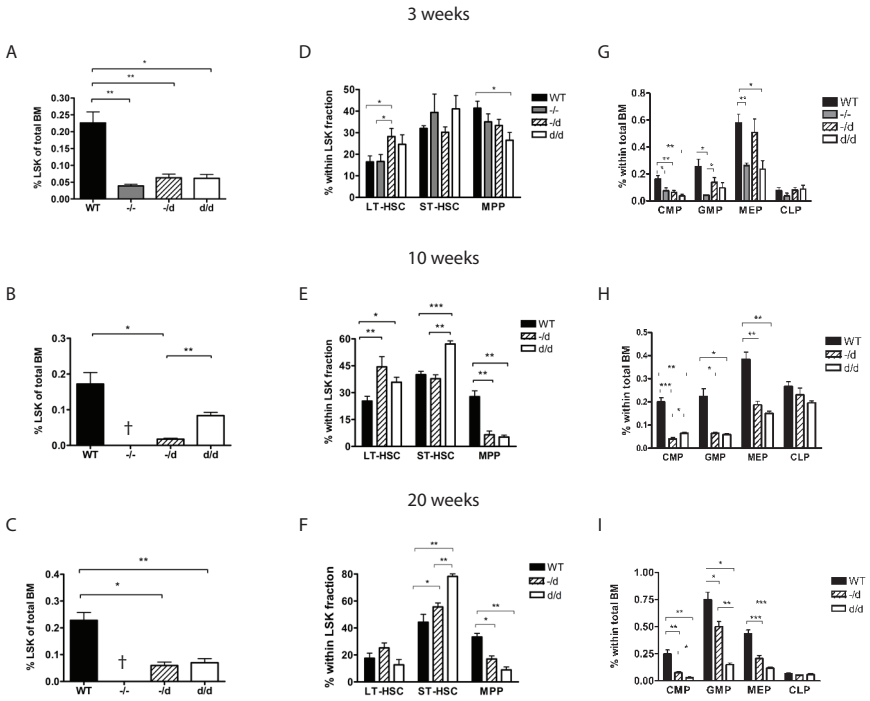

Figure 3: Ercc1 levels influence the composition of the stem and progenitor cell pool.

*Whole BM suspensions were stained with surface antigen specific antibodies for hematopoietic stem cells. (A,D,G) Lin- Sca1+ cKit+ cells as percentage of total bone marrow cells. (B,E,H) Distribution of stem cells within the LSK fraction (LT-HSC (CD34- CD135-), ST-HSC (CD34+ CD135-) and MPP (CD34+ CD135+)). (C,F,I) Distribution of progenitor cells within total bone marrow. Common Myeloid progenitors (CMP Lin- Ckit+CD34+CD16/CD32intermediate), Granulocyte-Monocyte progenitors (GMP, Lin- Ckit+CD34+CD16/CD32high), Megakaryocyte-Erythroid progenitors (MEP, Lin- Ckit+CD34-CD16/CD32low) and Common Lymphoid progenitors (CLP, Lin-CD127+Sca1/Ckit intermediate). Mean percentages are plotted; error bars indicate standard deviation. * indicates p≤0.05, ** indicates p≤0.01 and *** indicates p≤0.001.*

3.3 Ercc1 deficiency impairs colony formation by hematopoietic progenitors

To assess the colony forming potential of hematopoietic progenitors, bone marrow suspensions were plated in semi-solid medium supplemented with lineage-specific cytokines. At 3 weeks of age the number of erythroid (BFU-E, Figure 4A), granulocytic (CFU-G, Figure 4B) and granulocytic-macrophage colony forming cells (CFU-GM, Figure 4C) were significantly reduced in all Ercc1 deficient models relative to *wt* (Figure 4A-C).

Similar results were obtained in BM of 10 and 20 week old mice; *Ercc1*$^{-/d}$ BM formed no BFU-E colonies (Figure 4D,G), no CFU-G colonies (Figure 4E,H) and only 31% of CFU-GM colonies compared to *wt* (Figure 4F,I). In *Ercc1*$^{d/d}$ BM the percentages were 0%, 0% and 35% of *wt* respectively. These results imply that the residual Ercc1 activity in *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ mice is not sufficient to support BFU-E or CFU-G colony formation, whereas CFU-GM colony outgrowth is only partly restored.

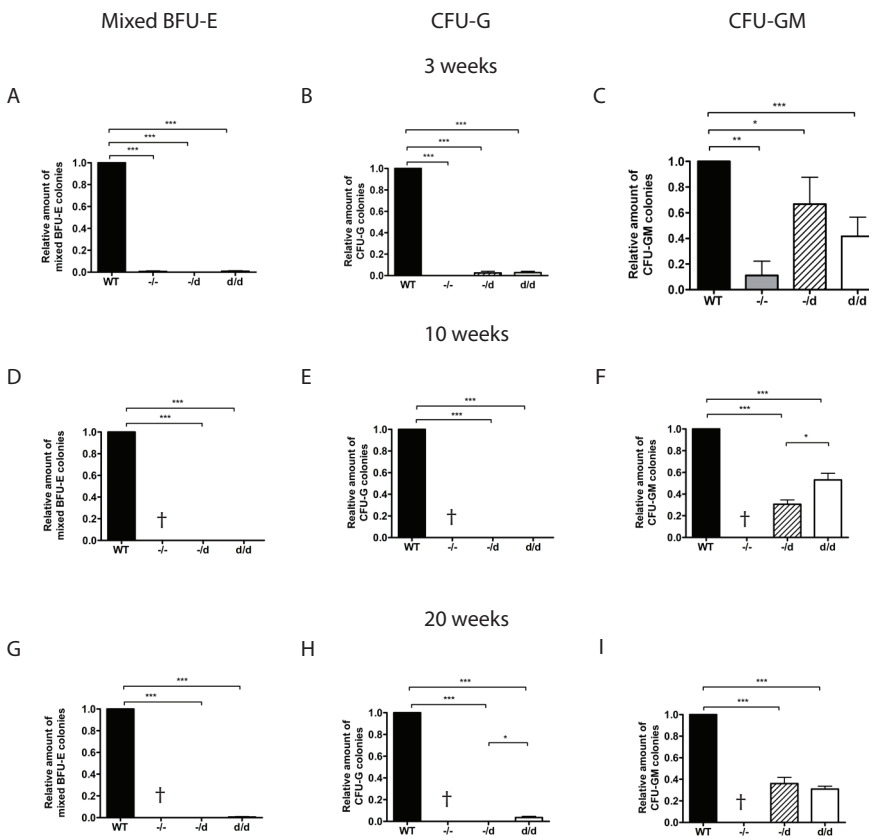

Figure 4: Colony forming potential of bone marrow progenitors is affected in hypomorphic *Ercc1* models.

*BFU-E, CFU-G and CFU-GM colonies per 5x10^4 bone marrow cells derived from (A-C) 3 week old Ercc1$^{-/-}$ (n=6), Ercc1$^{-/d}$ (n=7), Ercc1$^{d/d}$ (n=4) and wt mice (n=7) (D-F) 10 week old Ercc1$^{-/d}$ (n=3), Ercc1$^{d/d}$ (n=3) and wt mice (n=3) (G-I) 20 week old Ercc1$^{-/d}$ (n=3), Ercc1$^{d/d}$ (n=6) and wt mice (n=8) . Error bars indicate standard deviation. * indicates p≤0.05, ** indicates p≤0.01 and *** indicates p≤0.001.*

4. DISCUSSION

The Ercc1/Xpf endonuclease complex acts downstream of the Fanconi pathway in ICL repair [1-2]. The hematopoietic defects in Ercc1-deficient mice are reminiscent of the hematopoietic defect of FA patients [22]. Loss of HSC is seen when FA mice are challenged with Mitomycin C [23-24]. It mostly takes several years before FA patients develop anemia. The fact that most mouse models lacking Fanconi genes fail to display overt BM failure may reflect the time it takes to develop anemia. An important factor in the onset of BM failure and leukemia development may be the level of residual DNA repair activity. We employed Ercc1-deficient mouse models to show progressive loss of the number of hematopoietic stem and progenitor cells dependent on Ercc1 activity. Remaining progenitors were compromised in their *in vitro* proliferation capacity, which was similarly severe in *Ercc1*$^{-/-}$, *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ mice.

4.1 Reduced competitiveness of Ercc1-deficient hematopoietic cells

The conditional knock out model showed that a small percentage of hematopoietic stem- and progenitor cells in which the floxed *Ercc1* allele did not recombine outcompeted the Ercc1-deficient cells in which Cre-driven deletion had occurred. This progressive loss of Ercc1-deficient hematopoietic cells resembles what has been found in a small fraction of FA patients. In some FA patients a mutation was reverted, or two mutated alleles recombined and yielded an unaffected allele. Such a naturally corrected hematopoietic stem cell is able to out-compete the hematopoietic cells with two defective alleles resulting in the restoration of BM cellularity. In these mosaic patients cells fibroblasts retained two mutated alleles [25]. The conditional knock out mice that we used here underscores that defective ICL repair mainly affects continuously regenerating tissues such as BM and blood hematopoietic cells. It is also in the continuously proliferating bone marrow compartment that few cells with an intact allele can outcompete cells that lack a functional FA pathway.

4.2 Reduced hematopoietic reserves with normal peripheral blood levels

The hematopoietic defect in Ercc1-deficient mice, and in FA is specifically associated with DNA crosslinks that stall the replication fork. The inability to repair spontaneous DNA damage limits stress-hematopoiesis by diminishing the ability of HSCs to proliferate and self-renew. During embryo development and in young mice (<3 weeks) the HSC compartment is continuously expanded, whereas HSC become largely quiescent in adult mice [26-27]. These quiescent HSC are less sensitive to replication-coupled DNA damage repair defects. Progenitor cells have a higher proliferation rate compared to HSC and are therefore more prone to DNA damage both during development and in adult mice. Accordingly, we found that LSK numbers are 3- to 5-fold decreased compared to their *wt* littermates in *Ercc1* deficient mice. At 3 weeks of age the distribution within the LSK compartment hardly shows a tendency towards more primitive cells, most likely because all compartments contained proliferative cells. At 10 and 20 weeks of age, when the mice are adult, there is a significant shift towards the more primitive cells in the LSK compartment in the *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ compared to their *wt* littermates, indicating that maintenance of the LT-HSC fraction is less sensitive to DNA damage than the maintenance of the proliferative MPP fraction [28].

However, the mice did not develop overt anemia, and peripheral blood contained near normal amounts of red and white blood cells. This is most likely due to compensatory mechanisms controlled by a network of cytokines and hormones: Only small and transient alterations in local- and/or systemic concentrations will be needed to maintain or restore homeostasis. Notably, Epo serum concentrations were normal in Ercc1 deficient mice (data not shown), but this result was expected given that the mice were not anemic and Epo production in the kidney is activated by hypoxia.

Because cell numbers in peripheral blood are hardly affected, the hematopoietic defect in *Ercc1*$^{-/d}$ mice does not represent overt BM failure but can be regarded as a situation prone to such overt BM failure. Also in FA patients, reduced stem cell numbers precede overt BM failure and leukemia development [29-30]. When challenged for regeneration following insult, the Ercc1-deficient stem- and progenitor cells lack the robustness to do so. Analysis of BM and leukemogenesis in FA and in FA mouse models show that hypoplasia precedes leukemic transformation [31-32]. Hypoplastic compartments are most at risk for leukemic transformation [33]. FA patients

mainly develop acute myeloid leukemia (AML) and only very rarely acute lymphoid leukemia (ALL) [34]. In the Ercc1 models we also found that the myeloid compartment is affected by Ercc1 deficiency while the CLP compartment is hardly affected. Therefore, the hypomorphic Ercc1 mice may be a very useful model to study BM failure mechanisms and subsequent leukemogenic transformation in FA.

4.3 Comparison of the hematopoietic phenotype of Ercc1$^{-/-}$, Ercc1$^{-/d}$ and Ercc1$^{d/d}$ mice

In myeloid and erythroid colony forming assays *Ercc1* deficient progenitors show a 50% (on GM-CSF) to a 100% (on EPO/SCF or G-CSF) decrease in colony numbers. In *Ercc1*$^{-/d}$ and *Ercc1*$^{d/d}$ mice the decrease in colony numbers was not significantly different from those in *Ercc1*$^{-/-}$ mice. This implies that low levels of functional protein cannot repair the damage inflicted by the rapid proliferation which occurs in these assays. Flow cytometry measurements indicated that the decrease in myeloid and erythroid colony forming cell numbers was only moderate in the *Ercc1* deficient models at 3 weeks of age. Thus, the progenitors are present and they are able to generate progeny *in vivo*, but not *in vitro*. *In vitro* conditions challenge the proliferation capacity more than the *in vivo* condition and may be more mutagenic (*e.g.* due to higher oxygen levels).

5. ACKNOWLEDGEMENTS

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4

Critical role of p53 in interstrand-crosslink induced bone marrow failure and associated leukemic transformation

Jurgen R. Haanstra[a], Judith Verhagen-Oldenampsen[a], Paulina M.H. van Strien, Marijke Valkhof, Björn Schumacher, Ivo P. Touw and Marieke von Lindern

[a]These authors contributed equally

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Abstract

Defective DNA repair is an important factor in aging and cancer. In the hematopoietic system, bone marrow failure indicates a high risk to develop acute myeloid leukemia that is rarely associated with recurrent translocations. The mechanisms that drive leukemia from bone marrow failure, however, are largely unknown. Mice lacking the endonuclease Ercc1 show severe loss of hematopoietic stem- and progenitor cells (HSPC) due to defective repair of interstrand crosslinks (ICLs). In this study we investigate whether the tumor suppressors encoded by the *Trp53* or *Cdkn2a* loci are involved in the loss of HSPC from bone marrow and the associated leukemogenesis risk. Loss of *p53*, but not loss of the *Cdkn2a* locus, rescued bone marrow HSPC levels and colony formation, but it aggravated NER-dependent segmental progeria. In line with the dominant role of p53 in the hematopoietic phenotype, Ercc1-deficient bone marrow cells that are heterozygous for *p53* were prone to develop leukemia.

INTRODUCTION

Hematopoiesis is dependent on the lifelong maintenance of hematopoietic stem- and progenitor cells (HSPCs), which requires tight genome surveillance to prevent outgrowth of mutated clones. The cellular response to DNA damage consists of the recruitment of damage-specific DNA repair enzymes, the care-takers, and the activation of proteins that prevent the replication of damaged DNA, the gate keepers [1]. Examples of gate keepers are the cell cycle inhibitors Chk1 and Chk2 (Checkpoint homolog-1 and -2), the *Cdnk2a* (cyclin-dependent kinase inhibitor 2A) locus encoding $p16^{Ink4A}$ and $p14^{ARF}$/$p19^{Arf}$, and p53 [1-2].

The endonuclease Ercc1 is involved in global and transcription coupled nucleotide excision repair (NER), and in the excision of interstrand crosslinks (ICL). Mice lacking Ercc1 show severe segmental progeria due to defective NER, and premature loss of hematopoietic stem cells due to defective ICL repair [3, 20], chapter 3. Growth retardation of *Ercc1*$^{-/-}$ mice is due to suppression of the somatotrophic growth axis, reduced Igf-I signaling, and subsequent reduction of energy metabolism [4-5]. Growth retardation is independent of the classical gatekeepers *Trp53*, encoding p53, and the *Cdnk2a* locus encoding $p16^{Ink4A}$ and $p14^{ARF}$/$p19^{Arf}$ [4-6] Schumacher, unpublished). We previously observed that loss of HSPC is not due to the severe progeria as it occurs in *Xpa*$^{-/-}$*Csb*$^{-/-}$ mice. Instead, loss of HSPC may be due to cellular senescence and may depend on activation of p53 and expression of $p16^{Ink4a}$, events known to result in loss of tissue stem cells and tissue degeneration. Whether individual cells undergo cell cycle arrest, senescence or apoptosis depends on the activation level of the gatekeepers and persistence of the damage [7-9]. p53 and $p16^{Ink4a}$ have distinct roles in senescence [10]. In the hematopoietic system, however, activation of both gatekeepers has been associated with reduced proliferation of the stem cell pool, and loss of either gatekeeper is associated with leukemia. Loss of Atm leads to $p16^{Ink4a}$-dependent loss of hematopoietic stem cells (HSCs) [11], and methylation and silencing of *Cdkn2a* is commonly found in AML [12]. Activation of p53 restricts self-renewal of hematopoietic stem cells [13-15]. Mutations in *Trp53* are found in 10-15% of AML cases at diagnosis and are associated with aggressive disease and complex karyotypes [16-18]. Of note, hematopoietic progenitors lacking $p16^{Ink4a}$, $p19^{Arf}$ as well as p53 were reported to gain repopulation capacity [19]. However, despite their documented roles in the regulation of HSPCs expansion, it remains unclear whether either or both the *cdkn2a* locus and p53 are involved in the BM failure that arises from persistent ICLs, such as seen in Fanconi Anemia.

We used Ercc1-deficient mice to study how persistent ICL cause loss of BM HSPCs. The reduction of HSPCs, but not the growth defect and liver degeneration, was reverted by loss of *Trp53* in Ercc1-deficient mice. In contrast, loss of the *Cdkn2a* locus did not restore HSPC levels. Ercc1-deficient BM cells heterozygous for *Trp53* (*Trp53*$^{+/-}$) give rise to leukemia when transplanted in *wt* recipients. These data establish that hematopoiesis is particularly sensitive to ICL-induced p53 activation resulting in a reduction of the stem cell pool. Moreover, p53 heterozygosity in ICL-sensitive HSPCs predisposes to leukemia. In addition, the selective sensitivity of HSPCs to ICL suggests that the specific hematopoietic phenotype of FA is not due to functions of FA proteins distinct from DNA repair.

RESULTS

Ercc1$^{-/d}$ *HSPCs have reduced long term repopulation potential*

We recently showed that HPSCs are reduced in bone marrow of *Ercc1*$^{-/d}$ mice throughout their lifespan [20]. However, *Ercc1*$^{-/d}$ mice retain normal peripheral blood cell values. To study the long-term cell-intrinsic effects of Ercc1 deficiency and the associated ICL repair defect on hematopoiesis, *Ercc1*$^{-/d}$ FL or BM cells were transplanted into sublethally irradiated recipients. Recipients of *Ercc1*$^{+/-}$ control cells survived with a sustained (> 1 year) and high (>80%) donor contribution. In contrast, engraftment of *Ercc1*$^{-/d}$ cells was partial and only 4 of 10 transplanted mice showed stable engraftment of *Ercc1*$^{-/d}$ hematopoietic cells 6 months after transplant (Figure 1). One of these mice developed donor-derived (*Ercc1*$^{-/d}$) leukemia 42 weeks post-transplant. Under similar conditions, none of the full *Ercc1* knockout (*Ercc1*$^{-/-}$) BM cells showed sustained engraftment (Figure 1) and neither did BM cells from Tie2-Cre-driven knockout mice, in which the *Ercc1* gene is conditionally disrupted in the HSPCs [21-23] (Chapter 3). These results show that a residual level of Ercc1 is essential for engraftment of hematopoiesis *in vivo*. Thus, loss of Ercc1 severely impairs the repopulation ability of Ercc1-deficient HSPC.

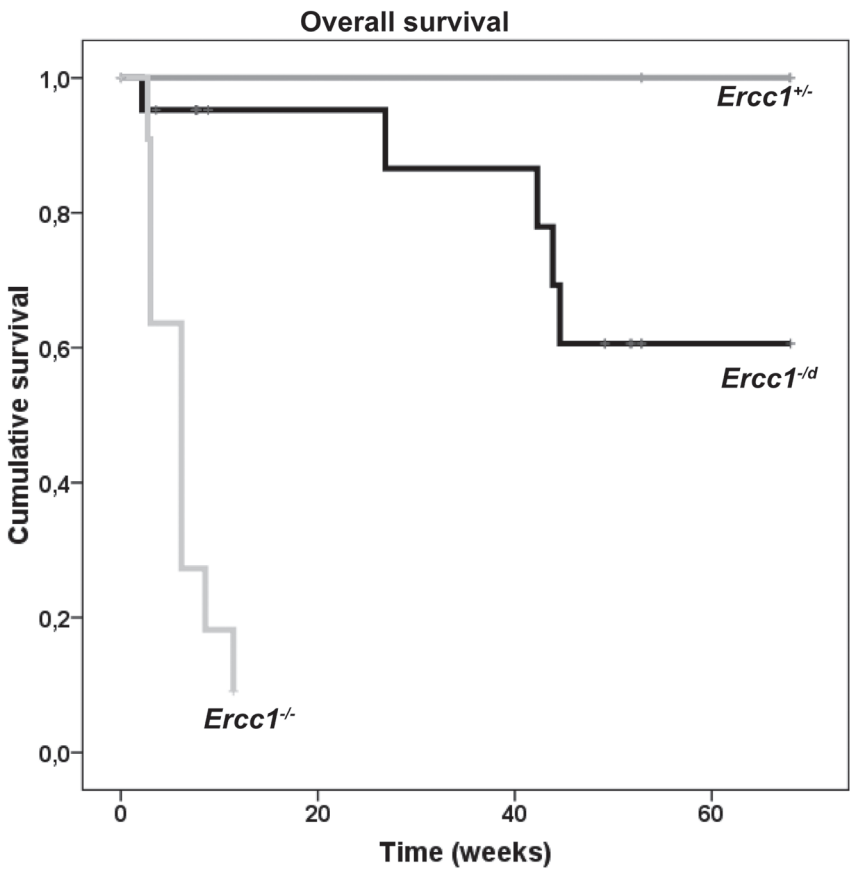

Figure: 1 Ercc1 deficient BM shows impaired repopulation capacity.
Fetal livers or BM was isolated from Ercc1$^{+/-}$ (wt), Ercc1$^{-/-}$ or Ercc1$^{-/d}$ E12.5 embryos or three week old animals. The total cell suspension from 1 fetal liver or 1x10^{7} BM cells were intravenously injected per recipient together with mature spleen cells for initial survival. Plotted are overall survival curves for the transplanted genotypes: Ercc1$^{-/d}$ (n=21), wt (n=6;) and Ercc1$^{-/-}$ (n=11)

Activation of p53, but not induction of $p16^{Ink4A}$-$p19^{Arf}$ represses hematopoiesis in $Ercc1^{-/d}$ mice

To determine which gatekeepers are involved in the reduced stem-and progenitor pool of Ercc1-deficient BM we first analyzed expression of the *Cdkna2* locus and *Trp53* as well as downstream target genes in the BM of 20-week old animals (Figure 2). From the *Cdkn2a* locus, the expression of $p16^{Ink4a}$ was 5.0-fold and expression of $p19^{Arf}$ 5.5-fold increased in $Ercc1^{-/d}$ BM. *Trp53* mRNA levels were unaltered, but the p53 target genes *PUMA* and *NOXA* were elevated 4.2-fold and 2.1-fold, respectively.

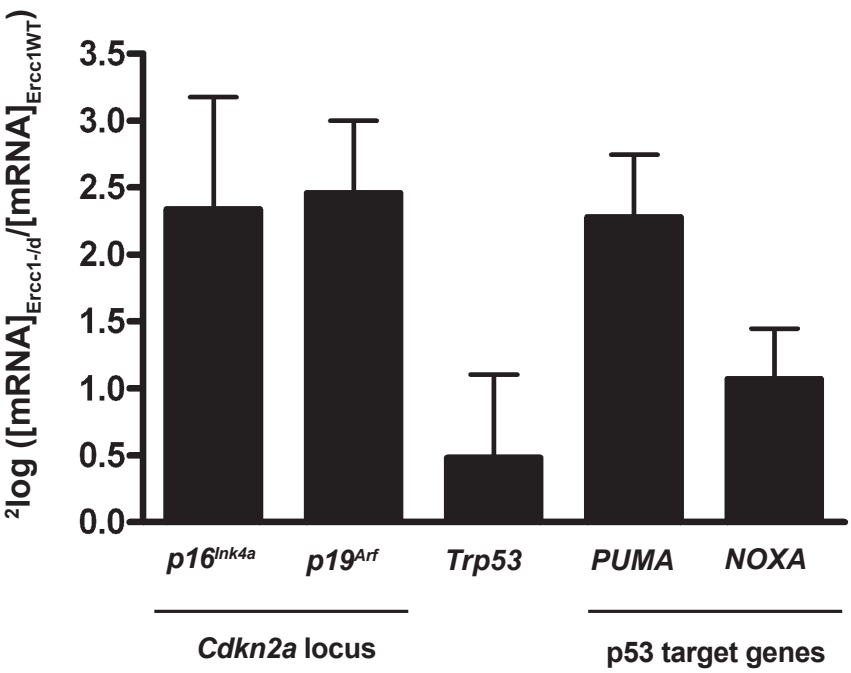

Figure 2: mRNA levels of *Cdkn2a*, *Trp53* and downstream targets in BM of 20-week old mice.

BM of three $Ercc1^{-/d}$ and three $Ercc1^{+/d}$ mice at 20 weeks of age were analyzed for expression of the transcripts indicated by qPCR. Graph shows the 2log of the fold-change in transcript levels of $Ercc1^{-/d}$ compared to $Ercc1^{+/d}$. Error bars indicate standard deviations (see also Table 3).

To investigate to what extent induction of $p16^{Ink4a}$, $p19^{Arf}$ or p53 contributes to BM failure in *Ercc1*$^{-/d}$ mice, *Ercc1*$^{-/d}$ mice were crossed with *Cdkn2a*- and *Trp53*-deficient mice [24-25]. The number of HSPC was less than half in BM of 10 week old *Ercc1*$^{-/d}$ mice compared to Ercc1 proficient littermates both in presence and absence of the *Cdkn2a* locus (Figure 3A). The number of CMP (common myeloid progenitor) and MEP (megakaryocyte/ erythroid progenitor), 2-fold reduced in *Ercc1*$^{-/d}$ bone marrow, was not altered (Figure 3B-D). Colony formation by the remaining progenitors in *Ercc1*$^{-/d}$ BM was strongly reduced in presence of GM-CSF (Figure 3E) or absent in presence of G-CSF or Epo/SCF (Figure 3F,G), and remained low or absent upon loss of the *Cdkn2a* locus (Figure 3E-G).

Deletion of the *Trp53* locus reduced the survival of *Ercc1*$^{-/d}$ mice from 20 to 3 weeks. Therefore, these mice and respective controls were analyzed at 19 days of age. The fraction of LSK cells in the BM of *Ercc1*$^{-/d}$*Trp53*$^{+/-}$ was restored to *wt* levels (Figure 4A). The levels of progenitor cells in *Ercc1*$^{-/d}$ BM as measured by flow cytometry did not significantly change upon loss of *Trp53* (Figure 4B-D), but deletion of *Trp53* increased the number of GM-CSF-induced colonies 3.3-fold (Figure 4E), G-CSF-induced colonies 12-fold (Figure 4F) and Epo/SCF responsive colonies 25-fold (Figure 4G) compared to *Ercc1*$^{-/d}$ littermates. Thus, absence of p53, but not p16 or p19 restored hematopoiesis in *Ercc1*$^{-/d}$ mice, indicating that activation of p53, but not of p16 or p19 inhibits proliferation of hematopoietic cells upon defective ICL repair.

Loss of Trp53 aggravates segmental progeria in Ercc1$^{-/d}$ *mice*

While loss of Trp53 rescued the hematopoietic phenotype of the *Ercc1*$^{-/d}$ mice, this was not the case for other phenotypic features of these mice. First, in addition to their shortened lifespan, *Ercc1*$^{-/d}$*Trp53*$^{-/-}$ animals are on average 25% smaller than *Ercc1*$^{-/d}$ littermates (Figure 5A), whereas loss of *Cdkn2a* has no effect on animal weight of Ercc1-deficient animals (Figure 5B). Second, Ercc1-deficiency causes prominent liver failure and polyploidy of hepatocytes [26-27]. In livers of *Ercc1*$^{-/d}$*Trp53*$^{-/-}$ mice, the numbers of polyploidy hepatocytes, characterized by enlarged nuclei, were increased relative to *Ercc1*$^{-/d}$ mice (Figure 5C,D). These results show that activation of p53, while responsible for reduced HSPC numbers in *Ercc1*$^{-/d}$ mice, restricted accelerated segmental progeria and hepatocyte damage.

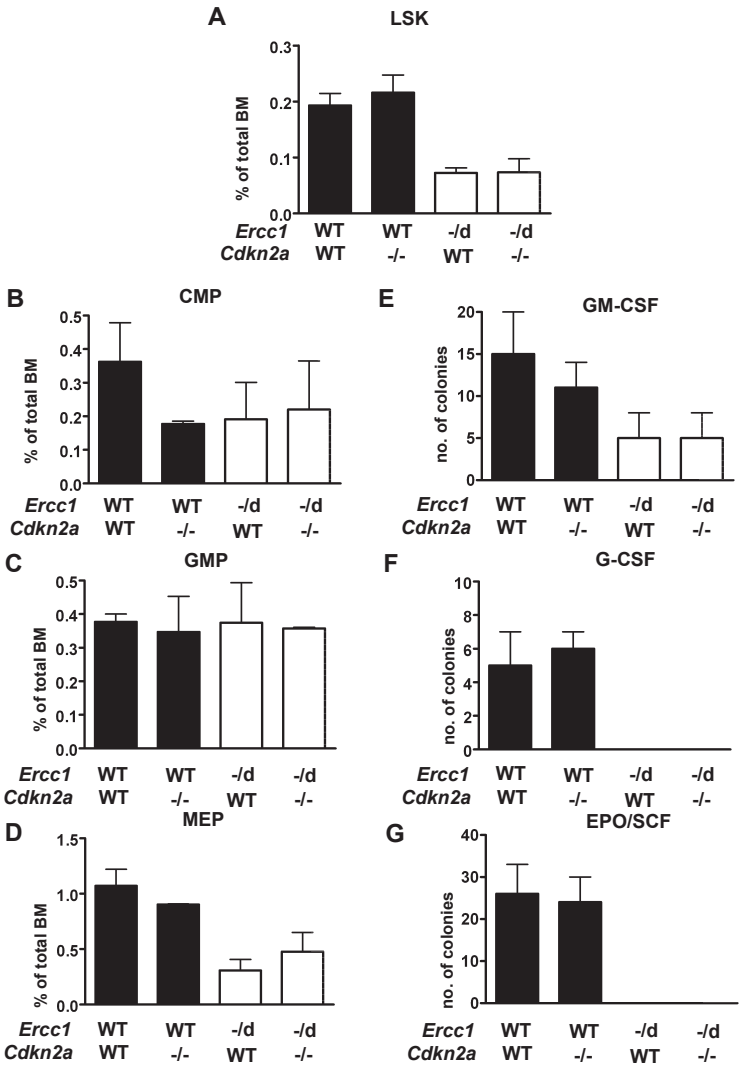

Figure 3: Depletion of *Cdkn2a* does not restore hematopoiesis in 10-week old *Ercc1*$^{-/d}$ mice.

(A-D) Frequencies of hematopoietic stem- and progenitor cells in total BM as determined by flow cytometry . The mouse genotype for Ercc1 and Cdkn2a loci is indicated below the bars. The mouse LSK (lineage marker negative, Sca1^{+} and C-kit^{+} cells) compartment contains the hematopoietic stem cells and multiponent progenitors. CMP: common myeloid progenitor, GMP: granulocyte/monocyte progenitor, MEP: megakaryocyte/erythrocyte progenitor. (E-G) Number of colonies counted 7-8 days after plating 5x10^{4} total BM cells in the presence of the growth factor(s) indicated. All bars show the mean of n≥3 animals. Error bars show standard deviations (see also Table 2).

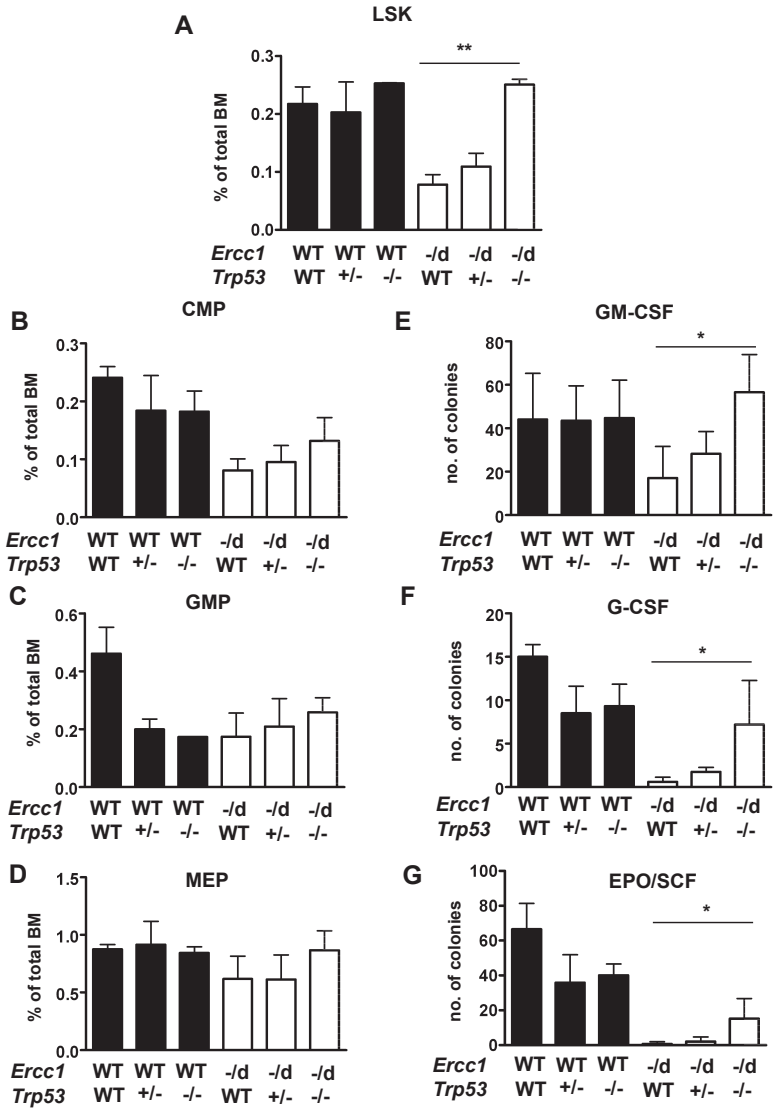

Figure 4: Depletion of *Trp53* restores hematopoiesis in *Ercc1*$^{-/d}$ mice.

*Mice were analyzed at postnatal day 19. (A-D) Frequencies of hematopoietic stem- and progenitor cells in total BM as determined by flow cytometry (see also legend Figure 2). The mouse genotype for Ercc1 and Trp53 loci is indicated below the bars.The mouse LSK compartment contains the hematopoietic stem cells and multiponent progenitors. CMP: common myeloid progenitor, GMP: granulocyte/ monocyte progenitor, MEP: megakaryocyte/erythrocyte progenitor. (E-G) Number of colonies counted 7-8 days after plating 5x10^4 total BM cells in the presence of the growth factor(s) indicated. All bars show the mean of n≥3 animals. Error bars show standard deviations. Data were analyzed by two-tailed student t-tests: *: p<0.05, **: p<0.001.*

Selective p53 dependence of cellular senescence in Ercc1$^{-/d}$ mice is cell type specific

To assay the proliferation capacity of hematopoietic progenitors, we expanded fetal liver-derived erythroblasts *ex-vivo*. In line with the *in vivo* results, loss of *Trp53* rescued *Ercc1*$^{-/d}$ erythroblast cultures from growth arrest while loss of *Cdkn2a* did not change erythroblast expansion (Figure 6A,C). However, when we cultured mouse embryo fibroblasts (MEF), both the deletion of the *Cdkn2a* locus and the *Trp53* restored proliferation of *Ercc1*$^{-/d}$ MEFs (Figure 6B,D). This indicates that the involvement of gatekeeper(s) in DNA damage responses is not only dependent on the type of DNA damage, but also on the cellular context.

Loss of Trp53 does not reduce MMC sensitivity and H2AX foci formation in Ercc1$^{-/d}$ mice

Because loss of p53 largely restored hematopoiesis in *Ercc1*$^{-/d}$ mice, we asked whether loss of p53 may also interfere with the response to ICLs induced by Mitomycin C (MMC). Erythroblasts were cultured in absence or presence of increasing concentrations MMC and the number of blasts was determined 48h following exposure to MMC. Erythroblasts cultured from *Ercc1*$^{-/d}$ fetal livers were hypersensitive to low concentrations of MMC. At 10nM only 25% of *Ercc1*$^{-/d}$ erythroblasts survived compared to untreated erythroblasts, whereas 80% of *wt* erythroblasts survived 10nM MMC for 48h (Figure 7A). *Trp53* deletion did not alter the sensitivity to MMC.

Stalling of the replication fork activates ATR, resulting in Histone 2Ax (H2Ax) phosphorylation within several kb of DNA flanking the stalled replication fork [28]. We determined the number of phospho-H2Ax foci in *Ercc1*$^{-/d}$ and *wt* erythroblast before and after 24h treatment with 0 to 40 nM MMC. The majority of *wt* erythroblasts had no foci, and treatment of *wt* cells with 40 nM MMC increased the amount of cells with foci to 50% of total (Figure 7B,C). In contrast, more than 75% of Ercc1-deficient erythroblasts already contained foci in the absence of MMC. Treatment with 40 nM MMC induced more than 5 foci in more than 60% of *Ercc1*$^{-/d}$ erythroblasts. Loss of p53 slightly reduced the number of cells with phosphorylated H2Ax, but did not abrogate the hypersensitivity of *Ercc1*$^{-/d}$ cells to MMC. These results demonstrate that *Ercc1*$^{-/d}$*Trp53*$^{-/-}$ erythroid cells remained hypersensitive to MMC and still elicited the DNA damage response.

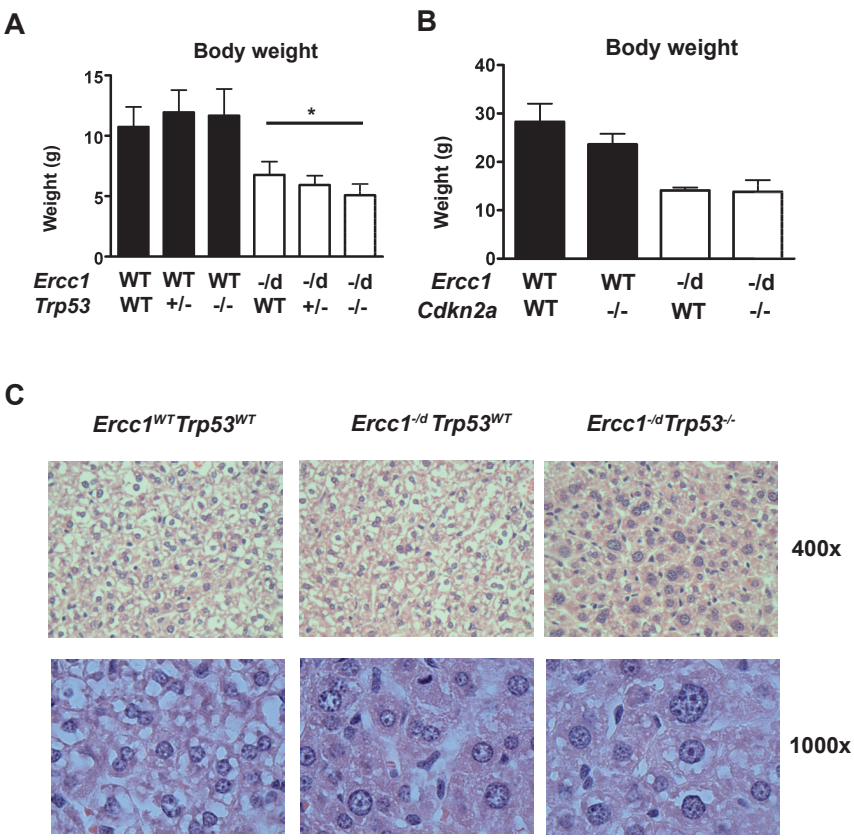

Figure 5: Deletion of *Trp53* does not restore growth defect and liver pathology of *Ercc1*$^{-/d}$ mice.

*Mice were analyzed at postnatal day 19 or week 10. (A) Body weight for 19 day old animals with the indicated genotypes. Error bars show the mean of n≥3 animals. Two-tailed student t-tests was done: *: p<0.05 (B) Body weight of 10 week old mice of the indicated genotypes. Error bars show standard deviations of n≥3 (C) Light microscopy images of paraffin-embedded livers stained with hematoxylin and eosin. The enlarged nuclei (arrows) indicate polyploidy, which is most severe in the Ercc1*$^{-/d}$*Trp53*$^{-/-}$ *mice. Top panel: pictures taken with 10x objective; lower panel: pictures taken with 100x objective.*

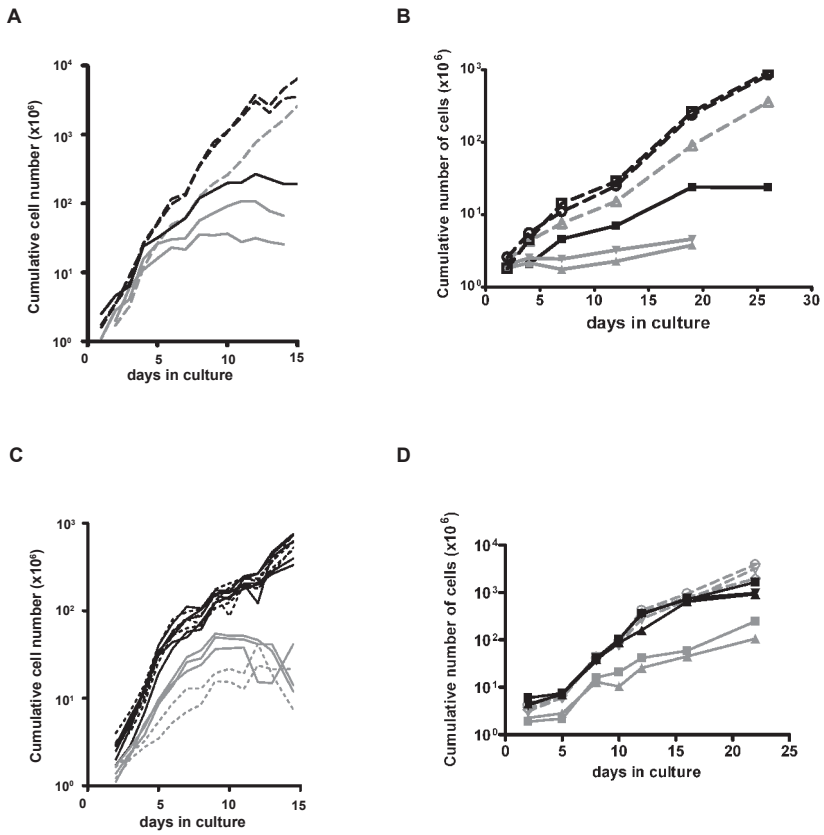

Figure 6: Selective *Trp53* dependence of cellular senescence in Ercc1$^{-/d}$ mice is cell type specific.

(A) Growth curves of erythroblast cultures. Black lines are Ercc1-proficient cultures, grey lines show Ercc1$^{-/d}$ cultures. Dashed lines show cultures (co-)depleted for Trp53. (B) Mouse embryonic fibroblasts (MEFs) and were grown from E12.5 embryos and were seeded in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FCS. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY-1, Scharfe-System, Reutlingen, Germany). Each line shows a separate experiment. Black lines show results from Ercc1-proficient MEFs, grey lines are Ercc1$^{-/d}$ MEFs. Dashed lines show cultures (co-)depleted for Trp53. (C) Growth curves of erythroblast cultures. Black lines are Ercc1-proficient cultures, grey lines show Ercc1$^{-/d}$ cultures. Dashed lines show cultures (co-)depleted for Cdkn2a. (D) Mouse embryonic fibroblasts (MEFs) and were grown from E12.5 embryos and were seeded in DMEM supplemented with 100 units/ml penicillin, 100 µg/ml streptomycin and 10% (v/v) FCS. Cell numbers and size distributions were determined daily, using an electronic cell counter (CASY-1, Scharfe-System, Reutlingen, Germany). Each line shows a separate experiment. Black lines show results from Ercc1-proficient MEFs, grey lines are Ercc1$^{-/d}$ MEFs. Dashed lines show cultures (co-)depleted for Cdkn2a.

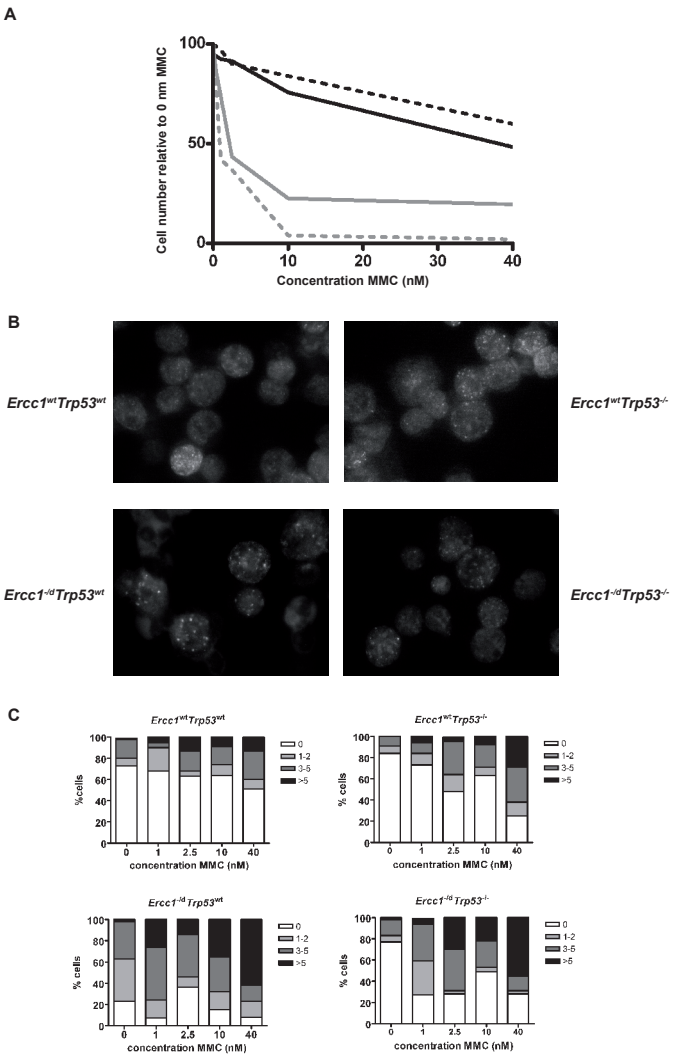

Figure 7: Effect of *Trp53* depletion on the DNA damage response in *Ercc1*$^{-/d}$ erythroblasts

(A) Number of erythroblasts (>8 μm) in cultures exposed for 48h to an increasing concentration of the DNA crosslinker Mitomycin C (MMC) compared to number of blasts at the start of the culture. Black lines: Ercc1-proficient cultures, grey lines: Ercc1$^{-/d}$ *cultures. Dashed lines show cultures (co-)depleted for Trp53 (B) Confocal pictures of cells exposed to 2.5 nM MMC for 16 h before they were fixed and stained with a phospho-specific H2AX antibody (C) Relative number of cells with a certain amount of H2AX-foci at an increasing concentration of MMC. Foci were counted in at least 300 cells and cells were divided in classes with a certain amount of foci per cell as indicated.*

Ercc1$^{-/d}$Trp53$^{+/-}$ HSPCs become leukemogenic in vivo

The activation of p53 inhibits expansion of hematopoietic stem- and progenitor cells and may concurrently prevent leukemic transformation of *Ercc1$^{-/d}$* cells. To investigate the leukemic potential of Ercc1-deficient HSC and early progenitors we transplanted BM cells into lethally irradiated *wt* recipients. The presence of the *Ercc1* knockout or delta allele in peripheral blood was analyzed by genomic real time PCR. We transplanted BM of *Ercc1$^{-/d}$Trp53$^{+/-}$* mice into lethally irradiated *wt* recipients (n=22) in 4 independent experiments. As controls we injected BM from *Ercc1$^{+/-}$Trp53$^{+/-}$* or *Ercc1$^{+/d}$Trp53$^{+/-}$* mice (n=21). Two mice that received *Ercc1$^{+/d}$Trp53$^{+/-}$* BM were taken out of the cohort because blood analysis failed to detect donor material. All other animals retained between 40-90% of donor cells in peripheral blood.

Mice showing signs of discomfort suggestive of hematopoietic disease (passive behavior, difficult thread, heavy breathing), were sacrificed and hematopoietic organs analyzed. From the 20 mice successfully transplanted with *Ercc1$^{-/d}$Trp53$^{+/-}$* BM cells, 2 survived 50 weeks post transplantation, whereas all control animals survived more than a year (Figure 8A). *Ercc1$^{-/d}$Trp53$^{+/-}$* -engrafted animals that were found dead (6/20) could not be diagnosed and appear as censored cases in the Kaplan Meier analysis (+ symbols in Figure 8A). Mice transplanted with *Ercc1$^{+/-}$Trp53$^{+/-}$* grafts that were sacrificed and analyzed as controls together with sick animals from the *Ercc1$^{-/d}$Trp53$^{+/-}$* cohort are also censored in this analysis. Diseased mice that were transplanted with *Ercc1$^{-/d}$Trp53$^{+/-}$*BM (12/20) suffered from an enlarged spleen (8/12) (Figure 8B and Table I) and/or thymus (6/12) (Table I). These symptoms, together with FACS analysis (Table I) and the accompanying anemia's were indicative of leukemia. The average latency of leukemia development was 29 weeks post-transplant. In all leukemia's analyzed, we confirmed donor origin in BM and blood samples by conventional or RT-PCR for the *Ercc1^{-}* and *Ercc1^{d}* allele. Finally, 7 leukemic samples were screened for expression of p53. *Trp53* expression was lost in 3 of 7 samples leukemia samples (Table I).

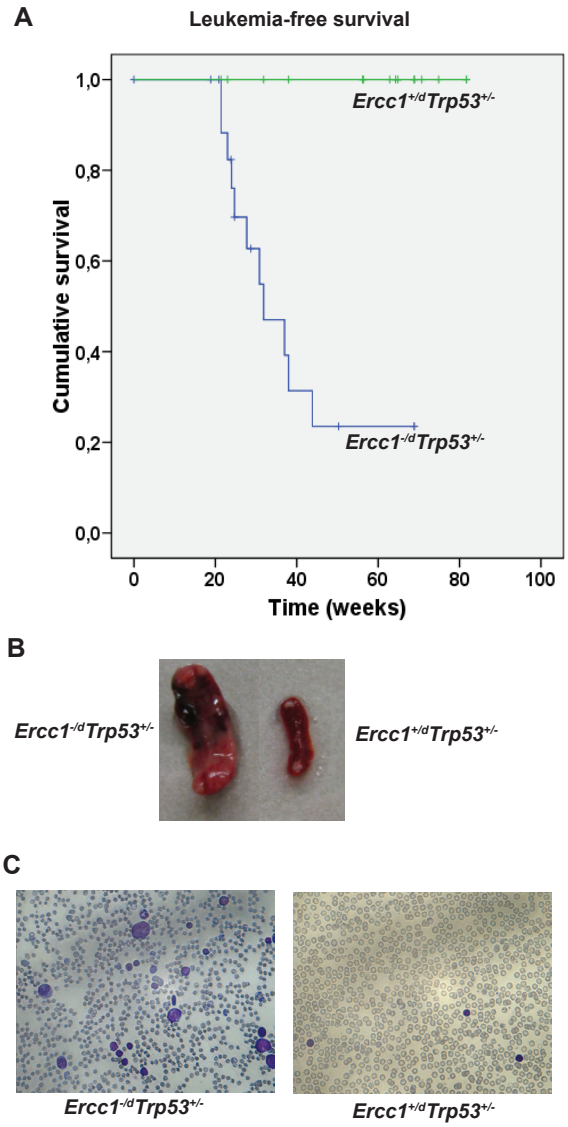

Figure 8: Transplantation of *Ercc1$^{-/d}$Trp53$^{+/-}$* BM cells yields leukemia.
(A) Kaplan-Meier survival curve for leukemia-free survival. Ercc1$^{-/d}$Trp53$^{+/-}$: n=20; Ercc1$^{+/d}$Trp53$^{+/-}$ n= 22. Cases that are censored in this analysis because they were sacrificed for analysis (Ercc1$^{+/d}$Trp53$^{+/-}$) or because they died of unknown cause (Ercc1$^{-/d}$Trp53$^{+/-}$) are marked by "+". (B) Spleen from an Ercc1$^{-/d}$Trp53$^{+/-}$ transplanted animal that got ill, and one from an Ercc1$^{+/d}$Trp53$^{+/-}$ recipient in the same experiment. (C) Blood smears from Ercc1$^{-/d}$Trp53$^{+/-}$ recipients that were ill. Cells were fixed and stained with May-Grunwald Giemsa. First panel was photographed with a 10x objective, 2nd and 3rd panel are photographed with a 100x objective.

DISCUSSION

The endonuclease Ercc1 is involved in the excision of lesions during global and transcription-coupled repair, and in the excision of ICL at the replication fork. Maintenance and repopulation ability of hematopoietic stem cells is particularly sensitive to ICL and impaired in Ercc1-deficient mice. This sensitivity results from activation of p53 and not from induced expression of p16 or p19 from the *Cdkn2a* locus. While loss of *Trp53* largely restored *in vivo* and *in vitro* hematopoiesis in *Ercc1*$^{-/d}$ mice, it aggravated segmental progeria caused by defective NER, which indicates that the function of gatekeepers depends on the type of DNA damage. In addition, gatekeepers act cell type specific, because loss of the *Cdkn2a* locus fails to rescue *Ercc1*$^{-/d}$ hematopoietic progenitors. Finally, we demonstrated that loss of one *Trp53* allele results in an increased leukemogenic potential of *Ercc1*$^{-/d}$ BM cells upon transplantation in *wt* recipients.

Trp53 has a dominant role in the response of hematopoietic cells to ICLs

Deletion of *Trp53* restored the stem- and progenitor cell compartment, colony forming capacity of progenitors and *in vitro* expansion of erythroblasts. Deletion of the *Cdkn2a* locus, on the other hand, did not modify the *Ercc1*$^{-/d}$ hematopoietic phenotype. Interestingly, this also implies that *p19*^{Arf} is dispensable for p53 activity in the BM of *Ercc1*$^{-/d}$ mice. The pronounced role of p53 seems to be cell-type and DNA-damage specific. Within the hematopoietic compartment, loss of *p16*^{Ink4a} expression restored the repopulation ability of ATM-deficient BM [11] but not in *Ercc1*$^{-/d}$ mice. Cell type specificity is indicated by the observation that loss of *Cdkn2a* restored the *in vitro* expansion of *Ercc1*$^{-/-}$ MEFs, but not of *Ercc1*$^{-/-}$ erythroblasts. Cell type and DNA damage specificity was most obvious from the observation that the small, runted phenotype of *Ercc1*$^{-/d}$ mice, and aberrant liver morphology, was even aggravated by the loss of *Trp53*. The *Ercc1*$^{-/d}$*Trp53*$^{-/-}$ animals were smaller and died at 3 weeks of age, compared to *Ercc1*$^{-/d}$ animals that live up to 25 weeks. Most likely, a p53-mediated cell cycle delay in the *Ercc1*$^{-/d}$ mice allows for alternative DNA repair, which decreases the load of DNA damage and the suppression of the somatotrophic growth axes. Because suppression of the growth axes was shown to be *Trp53* and *Cdkn2a* independent ([4, 6]; Schumacher, unpublished), the DNA-damage induced growth retardation of *Ercc1*$^{-/d}$ mice was enhanced in the absence of *Trp53*. Thus, the same DNA repair deficiency initiates distinct responses in the different cells and tissues of the body. In addition, distinct types of damage activate alternative tumor suppressor mechanisms. This may explain why DNA repair deficiencies can result in specific disease syndromes including segmental progeroid syndromes that affect some but not all tissues.

Table 1: Features of leukemias from *Ercc1*$^{-/d}$*Trp53*$^{+/-}$ bone marrow.

No	Exp	time (weeks)	WBC (x10^9/L)	RBC (x10^{12}//L)	Spleen enlarged	Thymus enlarged	p53 mRNA	Immunophenotype
1	1	38.0	1.3	0.51	+			lin-Sca1+/lin-CD127+, CD19+ blasts
2		21.4	1.6	1.03	+			CD19+ blasts
3*		31.9	9.3	3.31	+	+	-	CD19+ blasts
4		21.4	31.7	3.67	+			CD19+ blasts
5	2	37.0			+		+	LSKs (1%), CD3+CD19+ in blood
6		23.0	86.9	1.52		+	+	Cd3+DP
7	3	24.0	n.d.	n.d.	+		+	T-cell leukemia CD8+ (no CD4+, but DP)
8	4	27.7	15.3	9.79	+	+	-	BM: no LSK or LK, 68% T-cell, 50% DP
9		24.7	7.4	9.23		+	-	
10		30.9	low	low			+	lin-ckit+CD19+, CD19+ cells in all organs
11		44.0	2.8	3.76	+	+		50% BM is lin-Ckit+CD71+, many LSKs (2.7%)

WBC: peripheral white blood cell count (normal range: 3-15x10^9/L), RBC: peripheral red blood cell count (normal range: 5-12x10^{12}/L), BM: bone marrow, DP: double positive T-cells, +: present, -: absent, n.d.: not determined

* This animal also had a neck neoplasm

Ercc1$^{-/d}$Trp53$^{+/-}$ BM is prone to leukemia development

To investigate leukemogenesis in an *Ercc1$^{-/d}$* background, we transplanted *Ercc1$^{-/d}$* and *Ercc1$^{-/d}$ Trp53$^{+/-}$* BM into *wt* hosts. Only BM cells deficient in Ercc1 and heterozygous for p53 transformed to leukemia. The leukemia's that arose from *Ercc1$^{-/d}$Trp53$^{+/-}$* BM were heterogeneous with respect to their phenotype. They were characterized by an enlarged spleen (8/12) and/or thymus (6/12) and "lymphoid" markers as CD3e and CD19 were detected in many cases. Blood parameters, however, differed substantially as did the type of cellular abnormalities that were found by FACS analysis. Loss of 1 allele of *Trp53* most likely increases the chance that a cell completely loses *Trp53* expression by mutation. Notably, in 3 out of 7 leukemia's the expression of Trp53 was completely lost. Although loss of *Trp53* facilitates leukemia development, it is in itself not sufficient. We observed that complete loss of *Trp53* did not alter (*i*) the direct response to ICLs at the replication fork (*e.g.* H2Ax phosphorylation), or (*ii*) growth factor dependence and differentiation capacity of *Ercc1$^{-/d}$Trp53$^{-/-}$* immortalized erythroblast cultures (data not shown). Thus, loss of *Trp53* facilitates mutagenesis in an Ercc1-deficient background by abrogating the senescence response, but does not directly contribute to factor-independent proliferation or block of differentiation. The future challenge will be to detect the molecular and cellular processes that directly cause the leukemic phenotype in this setting.

The presented data may have implications for understanding the pathogenesis of therapy related myelodysplastic syndrome (tMDS) and AML (tAML) that may occur secondary to cancer treatment with alkylating agents and topoisomerase inhibitors. Alkylating agents induce DNA crosslinks, and topoisomerase II inhibitors enhance sensitivity to crosslinkers [29]. Whereas the frequency of mutations in *TP53* is approximately 10% in de novo MDS and AML, it is 25-30% in tMDS/tAML [30]. p53 activates a plethora of genes involved in stress responses and it remains to be established which of these downstream targets are critical for controlling responses of HSPCs to sustained ICL damage, *e.g.* in Fanconi Anemia. Notably, mutations in p53 rarely occur in AML secondary to Fanconi Anemia. The Ercc1-deficient mouse strains appear useful models for identification of these genes and connected regulatory pathways. Next generation sequencing of the genomes of HSPCs in these models at progressive stages of BM failure and transit to (pre-)leukemic stages is expected to provide further insights in these issues.

EXPERIMENTAL PROCEDURES

Animals

Ercc1$^{+/d}$, *Ercc1*$^{+/-}$ [31], *Cdkn2a*$^{+/-}$ [24] *Trp53*$^{+/-}$ [25], and *wt* littermates were kept in a background of C57/Bl6 and FVB/n at the Animal Resource Center (ErasmusMC). Experimental animals were generated as F1 in a mixed background of C57/Bl6 and FVB/n, except for the Csb and Xpa mice that are C57/bl6 only. *Ercc1*$^{+/-}$ and *Ercc1*$^{+/d}$ mice displayed a wild type phenotype and were used as controls. All animal studies were approved by an independent Animal Ethical Committee.

Mouse blood was obtained by tail vein puncture, or by heart puncture after mice were sacrificed by CO_2 inhalation, and analyzed on an Animal Blood Counter (ABX diagnostics). BM cell suspensions were obtained by crushing femurs, tibia and sternum in HBSS supplemented with 5% (v/v) fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. BM, spleen and thymus were passed through a 70 μM filter (BD Falcon, Bedford, MA) to obtain single-cell suspensions.

Cell culture

Erythroblasts were cultured in StemPro34 (Invitrogen, Breda, the Netherlands) supplemented with 1U/ml erythropoietin (Epo, Ortho-Biotech, Tilburg, The Netherlands), 100 ng/ml stem cell factor (SCF, supernatant of CHO producer cells) and dexamethasone (10^6 M; Sigma) at a density of $1x10^6$/ml. Cell numbers and size distributions were determined using an electronic cell counter (CASY-1, Scharfe-System, Reutlingen, Germany). Mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) was dissolved in 50% DMSO/H_2O and added at concentrations indicated in the text.

Colony forming assays

BM cell suspensions were plated at a density of 50.000 cells per ml per dish in triplicate in methyl cellulose medium (Methocult M3234, StemCell Technologies SARL, Grenoble, France) containing huGCSF (0.1 μg/ml), muGM-CSF (0.1 μg/ml), or Epo (4 mU/ml) plus transferrin (0.3 mM), hemin (0.2 mM) and muSCF (0.1 μg/ml). Colonies containing 30 cells or more were scored after 7-8 days of culture.

Flow cytometry

Blood and single cell suspensions from BM, spleen and thymus were analyzed by flow cytometry using a BD™ LSR II Flow Cytometer System with FCS Express Diva software (BD Biosciences., San Jose, California), and analyzed by FlowJo (Tree Star, Inc., Ashland, Oregon). Antibodies are listed in Table 2. 7-AAD (Invitrogen, Breda, the Netherlands) selected life cells.

Selected subpopulation consisted of: LSK (lineage marker negative, Sca1$^+$, ckit$^+$), long-term hematopoietic stem cells (LT-HSC): LSK,CD34$^-$ Flt3$^-$, short-term (ST) HSC: LSK, CD34$^+$Flt3$^-$, multipotent progenitors (MPP): LSK,CD34$^+$Flt3$^+$ common myeloid progenitor (CMP): lin$^-$ckit$^+$CD34$^+$CD16/CD32^{low}, granulocyte-monocyte progenitor (GMP): lin$^-$ckit$^+$CD34$^+$CD16/CD32^{high} and megakaryocyte-erythroid progenitor (MEP): lin$^-$ckit$^+$ CD34$^-$ CD16/CD32^{low}. See Figure 9 for gating strategy. Percentages can be compared because in all experiments the ratio (mononuclear cells)/(animal weight) was constant over all genotypes at a given age (Figure 10). Data from 1x10^6 BM cells were recorded to ensure reliable analysis of small populations.

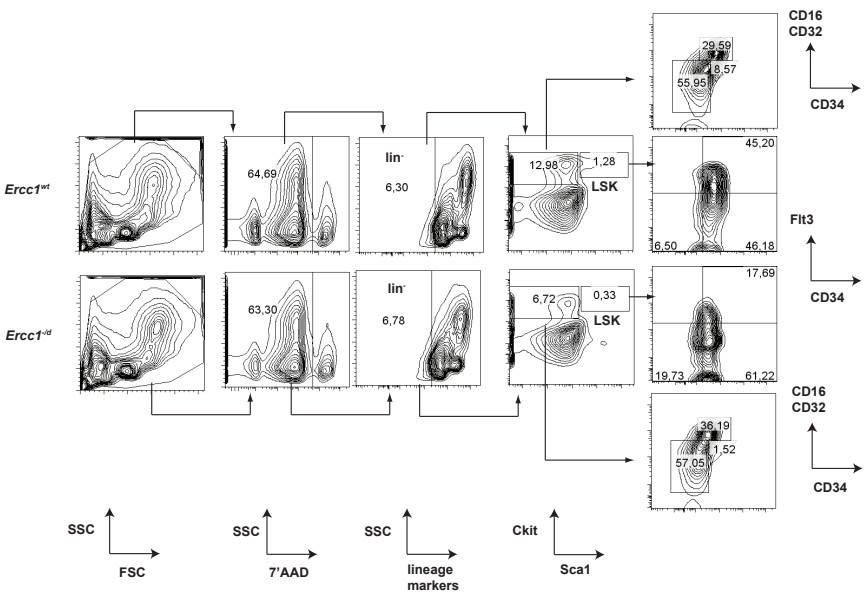

Figure 9: *Gating strategy for the LSK fraction and its subdivision and the committed progenitors. Example for mice ten weeks of age.*

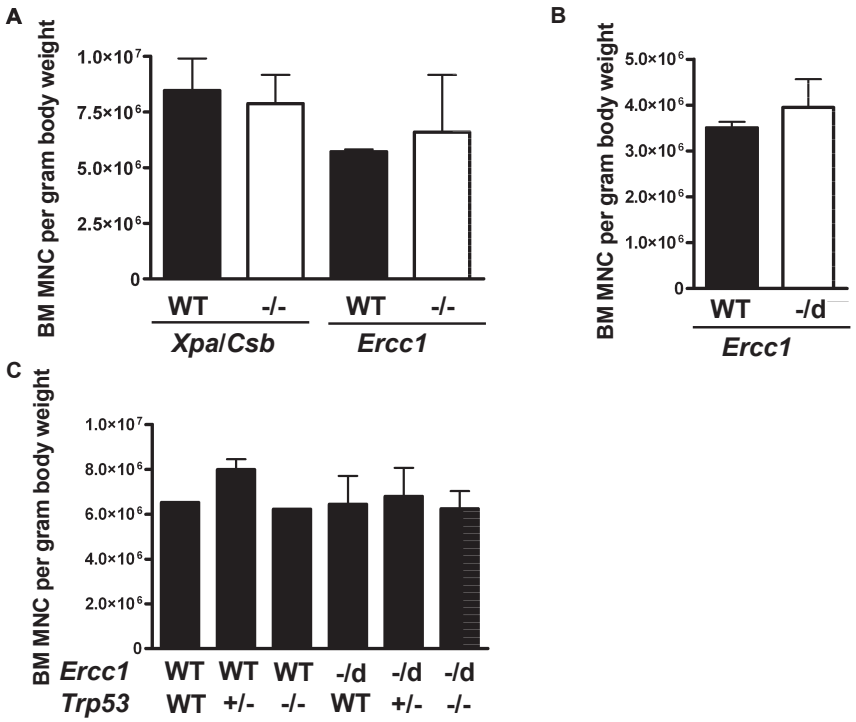

Figure 10: *The total number of BM mononuclear cells (MNC) obtained from two tibiae and the sternum divided by the body weight of the animals. Shown is the mean +/- standard deviation from n=3. When no error bar is given, the average of n=2 is shown. The ages of the mice , 10 weeks (A) and 21 days (B). Genotyping PCR and Q-PCR Genomic DNA was isolated from tail segments, or from blood (NucleoSpin Tissue XS, MACHEREY-NAGEL GmbH & Co). Genotypes were determined by PCR. Genomic Q-PCR used an Applied Biosystems 7900 instrument (Applied Biosystems, Weiterstadt, Germany) and SYBR Green PCR Master Mix (Applied Biosystems). Primers are listed in Table 3.*

Histology

Liver and spleen were embedded in Tissue-Tek O.C.T (Sakura Finetek, Zoeterwoude, Netherlands). 4 µm sections were made using a cryostat (Leica) and stained with hematoxylin and eosin. Slides were imaged on a Leica DMLB microscope equipped with Leica Application Suite 2.7.1.

Table 2: Antibodies used in Flow cytometry.

FACS antibody	clone	supplier
mouse biotinylated lineage depletion kit	145-2C11(CD3e), M1/70 (CD11b), RA3-6B2 (CD45R/b220), RB6-8C5 (Gr-1), Ter119 (Ter119)	BD pharmingen
Sca1-PE-cy7 (Ly-6A/E)	D7	ebioscience
CD34-pacific blue	RAM34	ebioscience
CD16/CD32-PE (FcγIII/II)	2.4G2	BD pharmingen
CD117-APC (Ckit)	2B8	BD pharmingen
CD127-pacific blue	A7R34	ebioscience
CD135-PE (Flt3)	A2F10.1	BD pharmingen
CD150-PE-cy5 (SLAM)	TC15-12F12.2	biolegend
streptavidin-APC-cy7		BD pharmingen
7'AAD		Invitrogen

All antibodies are anti-mouse

Immunofluorescence

Cytospins from cultured erythroblast were fixed in 2% (v/v) paraformaldehyde in PBS for 15 min at room temperature. After washing in PBST [0.1% (v/v) Triton X-100 in PBS] slides were blocked for 10 min in PBS+ (20mM Glycine and 75 mM BSA in PBS). Primary antibody was incubated overnight in PBS+ at 4°C in a humidified chamber, and washed in PBST and PBS+. Slides were incubated with secondary antibody for 1 hour at room temperature in a dark humidified chamber. Slides are mounted in VECTASHIELD HardSet mounting medium with DAPI (Brunschwig Chemie, Amsterdam, Netherlands) before visualization on an immunofluorescent microscope (Leica). For detection of phosphorylated γ-H2AX we used a rabbit polyclonal antibody (phospho S139) from Abcam and goat anti-rabbit IgG conjugated to Alexa Fluor 488 (Invitrogen).

Table 3: qPCR primers.

gene	Forward primer	Reverse primer
p16*	CGT ACC CCG ATT CAG GTG AT	TTG AGC AGA AGA GCT GCT ACG T
p19	CGT GAA CAT GTT GTT GAG GC	CGA ATC TGC ACC GTA GTT GA
p53	CCA TCC TGG CTG TAG GTA GC	AAT GTC TCC TGG CTC AGA GG
PUMA	CCA GAA ATG GAG CCC AAC TA	AAG GCT GGC AGT CCA GTA TG
NOXA	CCC ACT CCT GGG AAA GTA CA	AAT CCC TTC AGC CCT TGA TT
HPRT	AGC CTA AGA TGA GCG CAA GT	ATG GCC ACA GGA CTA GAA CA

* Taken from [32]

Transplantation assays

12-14 week old mice (F1 Fvb/n x C57/bl6) were irradiated with 9.2 Gy in a Gammacell-40 with two Cesium-137 sources. Two to four hours after irradiation mice were transplanted by tail vein injection with either a complete FL or $1x10^7$ total BM cells and $1x10^5$ WT spleen cells in 200 μl PBS containing 1% FCS. Animals were given ciprofloxacin in their drinking water post-transplantation. Kaplan-Meier survival curves were made in SPSS.

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Attenuated expansion and erythropoietin-induced signal transduction in Fanca and Fancg knockout erythroblasts is a p53-mediated response to defective repair of interstrand crosslinked DNA

Emile van den Akker, Henri J. van de Vrugt,
Judith Verhagen-Oldenampsen, Godfrey Grech,
Ivo Touw, Fré Arwert, Marieke von Lindern

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Abstract

Background

Fanconi anemia (FA) patients have a pronounced anemia and frequently develop bone marrow hypoplasia with a high risk of leukemic progression. The FA genes act in a pathway involved in DNA damage repair. Defects in the FA pathway result in hypersensitivity to interstrand DNA crosslinkers and oxidative stress leading to chromosomal instability and stress-induced senescence.

Design and methods

To investigate the cause of decreased erythropoiesis and insensitivity to Epo that develops in FA, we characterized factor-dependent expansion and Epo-induced signaling in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts.

Results

We show that erythroblast expansion capacity and Epo-induced phosphorylation of Stat5 and Erk1/2 are impaired in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts only when cultivated at atmospheric oxygen levels. SCF-induced signal transduction and Epo-induced phosphorylation of the Epo-receptor and Jak2 are not affected, suggesting that the signaling defect occurs distal of the Epo-receptor/Jak2 complex. Inefficient Epo signal transduction was also observed in *wild type* erythroblasts challenged with a sub lethal dose of the DNA crosslinker mitomycin C (MMC) in a p53 dependent manner and in nucleotide excision repair enzyme *Ercc1*$^{-/-}$ erythroblasts, functioning in the same ICL repair pathway as FA. In contrast, loss of nucleotide excision repair did not impair Epo-induced Stat5 phosphorylation.

Conclusion

Together the data suggest that deficient Epo-induced signal transduction in FA is secondary to p53 activation at the stalled replication fork, rather than a specific function controlled by Fanconi proteins.

BACKGROUND

Fanconi anemia (FA) is a genomic instability syndrome, characterized by developmental abnormalities, bone marrow failure, and cancer predisposition in particular acute myeloid leukemia (AML) and squamous cell carcinoma (SCC). Cells derived from FA patients show spontaneous chromosome breakage and are hypersensitive to oxidative stress and DNA crosslinking agents such as cisplatin and mitomycin C (MMC) [1-4]. FA is caused by bi-allelic inactivation of one of the fifteen genes that encode the so-called FA-pathway [5]. During S-phase interstrand crosslinks (ICL) stall the replication fork, activating ATR (Ataxia Telangiectasia and Rad3 related). ATR, in turn, activates numerous downstream pathways, including phosphorylation of H2AX, phosphorylation and stabilization of p53, phosphorylation and activation of Chk2 and p38. Activation of ATR also triggers the FA-pathway resulting in the efficient monoubiquitination of FANCD2 and FANCI by the ubiquitin ligase FANCL [6-10]. Monoubiquitination of FANCD2 and FANCI relocalizes these proteins to DNA-repair foci where they interact with the downstream Fanconi pathway components (FANCD1, FANCN, RAD51c (FANCO), FANCP, and FANCJ) and additional DNA repair proteins (e.g. BRCA1, FAN1, ERCC1, for review see [11]).

The FA pathway is embedded in a network that includes several proteins that are associated with instability syndromes or progeroid syndromes (e.g. Bloom syndrome, Nijmegen breakage syndrome and Ataxia Telangiectasia [5, 12-13]). Many of the proteins within these complexes have previously been identified in a BRCA1-associated genome surveillance complex (BASC). This complex stabilizes the replication fork at the site of DNA aberrations and allows for recruitment of the repair machinery [14-15]. The nucleotide excision repair enzyme ERCC1 is required for excision of the ICLs. Notably, mice lacking Ercc1 develop hypoplasia of the bone marrow compartment, similar to FA patients and Fancp/Slx4-deficient mice [16-17].

Mice lacking the FA core complex proteins Fanca, Fancc or Fancg display the characteristic sensitivity to DNA crosslinking agents (21-23). *Fancc*$^{-/-}$*Fancg*$^{-/-}$ double knockout mice and *Fancp*$^{-/-}$ mice develop bone marrow failure and AML [17-18]. Other Fanconi mouse models develop bone marrow failure when challenged: (*i*) *Fancc*$^{-/-}$ mice develop bone marrow hypoplasia when challenged with DNA crosslinking drugs [19], (*ii*) transplantation experiments showed that *Fancc*$^{-/-}$ murine stem cells have a compromized repopulation capacity and an increased risk for leukemic transformation after 4 days of culture at atmospheric oxygen [20-21].

Besides their role in maintaining genomic stability, defects in the FA pathway also affect the signal transduction of specific receptors via unknown mechanisms. For instance, IL3- and IL6-induced growth and differentiation is reduced in CD34+ cells derived from *Fancc*$^{-/-}$ bone marrow compared to *wt* littermates [22-23]. In addition, hematopoietic cells derived from FA patients or *Fancc*$^{-/-}$ mice are hypersensitive to TNFα and IFNγ [24-26], while IFNγ-induced Stat1 tyrosine phosphorylation is severely reduced in the same model [27].

Erythropoiesis is critically dependent on the Epo-receptor and Epo-induced signal transduction. In synergy with stem cell factor (SCF), and glucocorticoids, Epo regulates proliferation of early erythroblasts [28-31]. Phosphorylation and nuclear location of Stat5 is an important pathway mediating Epo-dependent cell survival [32]. Indeed, Stat5 activation enables erythropoiesis in the absence of EpoR and Jak2 [33-34]. Notably, FA patients presenting with anemia have high Epo serum levels that fail to rescue anemia, suggesting a defect in Epo signal transduction [35-36].

To investigate the cause of decreased erythropoiesis and insensitivity to Epo that develops in FA, we characterized factor-dependent expansion and Epo-induced signaling in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts. Erythroblasts derived from *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ fetal livers show a progressive loss of expansion capacity after 10 days in culture at atmospheric oxygen. We found Epo-induced Stat5 and Erk1/2 phosphorylation to be severely reduced in an oxygen dependent manner in *Fanca*$^{-/-}$, *Fancg*$^{-/-}$ and *Ercc1*$^{-/-}$ erythroblasts. In addition, the crosslinking agent MMC caused decreased Epo-induced Stat5 and Erk1/2 phosphorylation in *p53*^{wt} but not in *p53*$^{-/-}$ cells. Loss of the *Cdkn2a* locus did not improve Epo signaling in presence of MMC. Together the data suggest that impaired Epo signaling in Fanconi anemia is part of a general cellular response to DNA damage dependent on p53. This also suggests that similar defects may occur in related DNA repair syndromes and upon aging.

DESIGN AND METHODS

Antibodies and growth factors

Rabbit polyclonal anti-Jak2 and mouse monoclonal anti-phospho-Stat5 (Tyr694-Stat5A; Tyr 699-Stat5B; P-Stat5) were obtained from Upstate Biotechnology (Massachusetts, USA). Rabbit polyclonal anti-Epo-receptor, c-Kit, p44/p42 MAP kinase and anti-PY99 mouse monoclonal antibody from Santa Cruz Biotechnology (Heidelberg, Germany); mouse monoclonal anti-phospho-p44/p42 MAP kinase (Thr202/Tyr204; P-Erk) from New England Biolabs (Frankfurt am main, Germany). Recombinant human Epo was a gift from Ortho-Biotech (Tilburg, The Netherlands), dexamethasone (dex) was purchased from Sigma (Zwijndrecht, The Netherlands) and human SCF was produced by CHO cells (supernatant).

Cell culture

Fanca$^{-/-}$ and *Fancg*$^{-/-}$ mice were previously described [37-38]. Primary fetal liver erythroblasts were obtained from day E12-13 fetal livers and cultured to a homogenous erythroblast pool in serum free conditions using StemPro-34 medium (Invitrogen, Breda, The Netherlands) supplemented with Epo (0.5U/ml), SCF (100ng/ml) and Dexamethasone (1mM, Sigma) as described previously [29]. Cell cultures were maintained at 1.5-3x10^6 cells/ml and a homogenous erythroblast culture was obtained within 4 days. All starvation/stimulation experiments were performed with cultures expanded for 6 days. In some experiments Mitomycin C (MMC; Kyowa Hakko Kogyo, Tokyo, Japan) was added to the cultures (concentrations indicated in the figure legends).

Immune-precipitations, Western blotting and electrophoretic mobility shift assays (EMSA)

Primary erythroid fetal liver cell cultures or bone marrow preparations were growth factor deprived for 4h in plain IMDM (Invitrogen). Erythroblasts (40-80x10^6/ml) were stimulated at 37°C with SCF (1mg/ml; 5'), or Epo (5U/ml; 10') as indicated in the figures. Cell lysis, immune-precipitations, SDS-polyacrylamide gel electrophoresis, Western blots, preparation of nuclear extracts and Stat5 mobility shift assay were all performed as described previously [39-40].

Flow cytometry

To measure Epo-receptor (EpoR) membrane expression, Epo was biotinylated following the biotin labeling kit protocol from Sigma (#1418165). Erythroblasts ($1.0x10^6$ per sample) were growth factor deprived for 4h in IMDM, washed, incubated with 0.5 U/ml BioEpo and stained as described [41]. To control for specific binding, a 10-fold excess of non-biotinylated Epo was added in parallel incubations.

To distinguish between live and dead cells, the cell cycle profile was determined. 0.5 to $1.0x10^6$ cells were fixed and permeabilized with ice-cold methanol (0.5 ml; 30') washed 2 times with Phosphate buffered saline (PBS) and incubated for 30' with 0.5% w/v RNAse A (Sigma) in PBS under constant shaking. DNA was stained with propidium iodide (2 μg/ml in PBS, Sigma) and fluorescence was measured by flow cytometry.

Real time PCR

cDNA synthesis and quantitative RT-PCR were performed using Taqman technology and Sybr-green detection of dsDNA as described [42]. The primer-sequences used for the amplification are given in Table 1. The CT-values of RI were used to normalize the values.

Table 1: ***primer sequences used in RT-PCR (5' → 3')***

Gene	direction	sequence
p21	forward	ACCAGAGGGAGCCTGAAGACT
	reverse	ACCAGAGGGAGCCTGAAGACT
Socs3	forward	TCAAGACCTTCAGCTCCA A
	reverse	TCTTGACGCTCAACGTGAAG
Spi2.1	forward	ACTGCCTTGGCCCTCCTGTC
	reverse	TGCCTGTGCTGATCTGTACC
BclXl	forward	TGGTCGACTTTCTCTCCTAC
	reverse	TCACTACCTGCTCAAAGCTC
Tubulin	forward	TGCAGCGTGCTGTGTGCATG
	reverse	TCCTCTCGAGCCTCAGAGAA
Gilz	forward	TGCGGAGTACCTCACAGAG
	reverse	TCCTTGTCAGCGAAAGTACCA
RI	forward	TCCAGTGTGAGCAGCTGA G
	reverse	TGCAGGCACTGAAGCACCA

RESULTS

Fanca$^{-/-}$ and Fancg$^{-/-}$ erythroblasts have a reduced expansion capacity

To examine expansion and survival of *Fanca$^{-/-}$* or *Fancg$^{-/-}$* erythroblasts under stress conditions, we studied *in vitro* expansion of erythroblasts using a model for stress-erythropoiesis [30-31, 43]. Fetal liver-derived erythroblasts from *wt* and knockout mice proliferated with comparable kinetics during the first 9 days of culture. However the proliferation capacity of *Fanca$^{-/-}$* and *Fancg$^{-/-}$* progenitors was severely reduced compared to *wt* from day 10 onward (Figure 1A, B). No increase in cell death was detected when the proliferative capacity of *Fanca$^{-/-}$* or *Fancg$^{-/-}$* cells declined (Figure 1C). Instead, the number of large blast-cells, able to undergo renewal divisions, decreased sharply around day 10 while the number of smaller cells was retained (Figure 1D and morphological analysis not shown). This indicates that *Fanca$^{-/-}$* and *Fancg$^{-/-}$* erythroblasts succumbed to differentiation 8 days earlier compared to wt littermates.

Treatment of *Fancc$^{-/-}$* mice with the DNA crosslinker MMC, showed that the bone marrow compartment is particularly sensitive [19]. To test if this sensitivity is reflected in *in vitro* expanded erythroblasts, we analyzed cell growth in presence of increasing concentrations of MMC. Expansion of *wt* cells was inhibited at 50nM MMC but *Fancg$^{-/-}$* progenitors were affected at 2nM MMC (Figure 1E). Similar results were obtained for *Fanca$^{-/-}$* cells (data not shown). The decrease in viable cells reflected the sensitivity for MMC (Figure 1F). Thus, *Fanca$^{-/-}$* and *Fancg$^{-/-}$* erythroblasts have a reduced expansion capacity and are extremely sensitive to genotoxic stress.

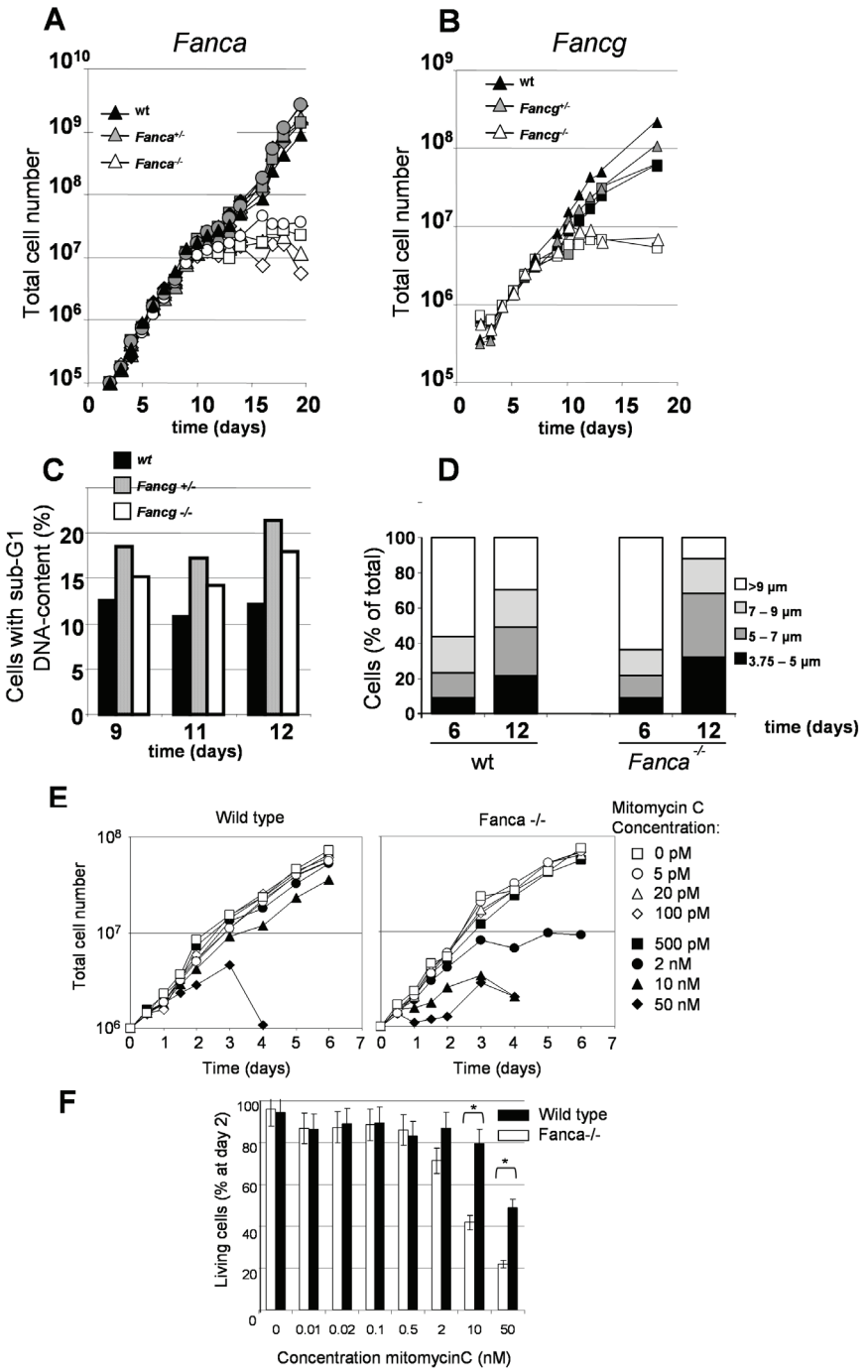
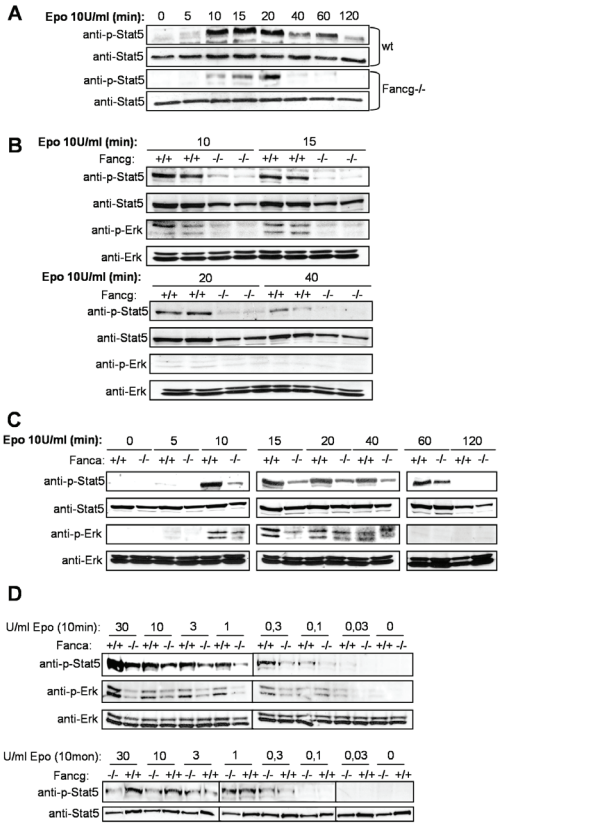

A
Fanca
Total cell number
wt
Fanca+/-
Fanca-/-
time (days)
B
Fancg
Total cell number
wt
Fancg+/-
Fancg-/-
time (days)
C
wt
Fancg +/-
Fancg -/-
Cells with sub-G1 DNA-content (%)
time (days)
D
Cells (% of total)
>9 µm
7 – 9 µm
5 – 7 µm
3.75 – 5 µm
time (days)
wt
Fanca-/-
E
Wild type
Fanca -/-
Total cell number
Time (days)
Mitomycin C Concentration:
0 pM
5 pM
20 pM
100 pM
500 pM
2 nM
10 nM
50 nM
F
Living cells (% at day 2)
Wild type
Fanca-/-
Concentration mitomycinC (nM)

Figure 1: The expansion capacity of Fanca$^{-/-}$ and Fancg$^{-/-}$ erythroblasts is reduced compared to wt progenitors.
*(**A**, **B**) Erythroblasts derived from wt (black symbols), heterozygous (grey symbols), Fanca$^{-/-}$ (**A**; open symbols) or Fancg$^{-/-}$ (**B**; open symbols) fetal livers were cultured in serum free expansion medium containing Epo, SCF and dex (symbols represent different littermates). Presented data are derived from 1 litter each and are representative for other litters analyzed.* **C***) Aliquots of the experiment described in B were taken on day 9, 11 and 12 of culture. Cells were fixed and stained with Propidium Iodine to determine the percentage of subG1 fragmented DNA fraction by flow cytometry, which is indicative for dead cells.* **D***) Relative cell numbers sorted for cell size (Casy cell counter) in a typical culture of Fanca$^{-/-}$ erythroblasts (see A). Only blastoid cells (>9 μm) are able to undergo expansion divisions. Mature erythrocytes generated in culture are at average 4.5 μm in size.* **E***) Erythroblasts from wild type and Fancg$^{-/-}$ fetal livers were expanded in presence of different concentrations of MMC as indicated. Total blasts are plotted against time in days.* **F***) 2 days following addition of MMC, cells were stained with PI and the percentage of living cells was determined by flow cytometry. Asterisks indicate statistical significance ($p<0.05$; student T-Test) between wt and Fanca$^{-/-}$ cells at a given MMC concentration.*

Epo-induced signal transduction is impaired

We next investigated whether the reduced expansion ability could be caused by impaired Epo and/or SCF signaling. We examined signaling in cells cultured for 6 days to ensure that we compare erythroblasts before the onset of Fanca or Fancg deficiency-induced differentiation and growth defects. Epo-induced phosphorylation of Stat5 and Erk1/2 was severely reduced and delayed in the *Fancg*$^{-/-}$ and *Fanca*$^{-/-}$ cells (Figure 2 A-C). Not only the phosphorylation, also expression of Stat5 is slightly reduced in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ progenitors when related to the Erk1/2 loading control. However, Epo-induced phosphorylation is reduced in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ cells also when the lower Stat5 expression is taken into account.

To examine if these signaling defects arise at more physiological concentrations of Epo (0.03 to 0.1 U/ml) we exposed erythroblast cultures to Epo concentrations ranging from 0 to 30 U/ml. At all Epo concentrations, the phosphorylation of Stat5 and Erk1/2 was decreased in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts compared to progenitors derived from *wt* littermates (Figure 2D).


A
Epo 10U/ml (min): 0 5 10 15 20 40 60 120
anti-p-Stat5
anti-Stat5
wt
anti-p-Stat5
anti-Stat5
Fancg-/-
B
Epo 10U/ml (min): 10 15
Fancg: +/+ +/+ -/- -/- +/+ +/+ -/- -/-
anti-p-Stat5
anti-Stat5
anti-p-Erk
anti-Erk
Epo 10U/ml (min): 20 40
Fancg: +/+ +/+ -/- -/- +/+ +/+ -/- -/-
anti-p-Stat5
anti-Stat5
anti-p-Erk
anti-Erk
C
Epo 10U/ml (min): 0 5 10 15 20 40 60 120
Fanca: +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/-
anti-p-Stat5
anti-Stat5
anti-p-Erk
anti-Erk
D
U/ml Epo (10min): 30 10 3 1 0,3 0,1 0,03 0
Fanca: +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/-
anti-p-Stat5
anti-p-Erk
anti-Erk
U/ml Epo (10mon): 30 10 3 1 0,3 0,1 0,03 0
Fancg: -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+
anti-p-Stat5
anti-Stat5

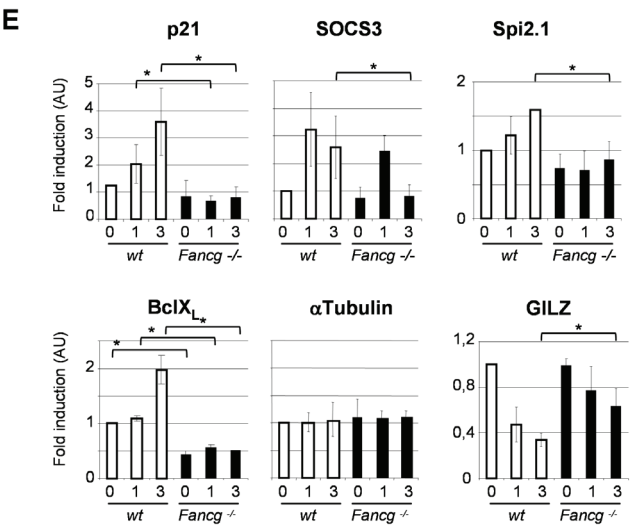

E
p21
SOCS3
Spi2.1
Fold induction (AU)
0 1 3 0 1 3
wt
Fancg -/-
BclX$_{L}$
αTubulin
GILZ
Fancg -/-

Figure 2: Epo-induced phosphorylation of Stat5 and Erk1/2 is reduced in Fanca$^{-/-}$ and Fancg$^{-/-}$ erythroblasts.

*(**A-C**). Wild type (wt or +/+), Fancg$^{-/-}$ (-/-) (A, B) or Fanca$^{-/-}$ (-/-) (**C**) fetal liver-derived erythroblasts were factor deprived for 4h and stimulated with Epo (5U/ml) for the indicated time points. Cells were lysed and whole cell lysates were analyzed by Western blotting using the indicated antibodies.* **D***) Wt (+/+), Fanca$^{-/-}$ (-/-) and Fancg$^{-/-}$ (-/-) fetal liver erythroblasts expanded for 6 days were factor deprived for 4h and stimulated with various concentrations of Epo (0-30U/ml) for 10'. Cells were lysed and whole cell lysates were analyzed by Western blotting. (**A-D**) Blots were stained with anti-phospho-tyrosine-Stat5a/b (p-Stat) or anti-phospho-Erk1/2 (p-Erk). As a loading control the same blots were re-stained with anti-total-Stat5a/b (Stat5) or anti-total-Erk1/2 (Erk) as indicated.* **E)** *Wt (white bars) and Fancg$^{-/-}$ (black bars) erythroblasts expanded for 6 days were factor deprived for 4h and stimulated with Epo. At the indicated time points (0, 1, 3h) aliquots were taken from the stimulated cells for RNA isolation. mRNA levels of p21, SOCS3, Spi2.1, Tubulin, Ribonuclease Inhibitor (RI) and GILZ were determined by real-time-PCR. Fold induction of Epo-induced genes was normalized against RI and is depicted compared to the specific RNA levels of non-stimulated wt cells. Tubulin is an additional control for the normalization of values on RI. Values represent mean and standard deviation of three experiments and at least 3 independent measurements per condition were performed. Asterisks indicate statistical significance ($p<0.05$; student T-Test) between wt and Fancg$^{-/-}$ cells at a given time point.*

Subsequently we investigated whether attenuated Epo-induced signal transduction modifies regulation of known Epo target genes. In accordance with previous results [42], Epo induced expression of e.g. *p21^{WAF}*, S*ocs3* (suppressor of cytokine signaling), S*pi2.1* (serine protease inhibitor 2.1) and repressed expression of *Gilz* (glucocorticoid induced leucine zipper) in *wt* progenitors. This regulation was absent (*p21, Spi2.1*) or reduced (*Socs3, Gilz*) in erythroblasts expanded from *Fancg$^{-/-}$* fetal livers (Figure 2E).

Signaling in Fanca$^{-/-}$ and Fancg$^{-/-}$ progenitors is specifically abrogated downstream of the EpoR/Jak2 complex

Impaired Epo-induced signal transduction could be caused by decreased EpoR membrane expression. However, EpoR membrane expression, estimated by the amount of Epo binding sites using biotinylated Epo was similar in *wt*, *Fanca$^{-/-}$* and *Fancg$^{-/-}$* cells (Figure 3A). EpoR signal transduction is dependent on the activation of Jak2, which phosphorylates the distal tyrosines on the EpoR (for review see [44]). Epo-induced phosphorylation of the EpoR was equally efficient in progenitors expanded from *Fanca$^{-/-}$*, *Fancg$^{-/-}$* and *wt* fetal livers (Figure 3B, C), and also Jak2 phosphorylation is unchanged

suggesting that the signaling defect occurs downstream of Jak2-mediated EpoR phosphorylation. To investigate whether signaling defects are specific to Epo-induced signaling or applicable to receptor signaling in general, SCF signal transduction was investigated. SCF-induced auto-phosphorylation of its receptor c-Kit and the phosphorylation of the downstream target Erk1/2 were not affected in *Fanca*$^{-/-}$ cells (Figure 3D, E).

Stat5 activation in vivo is unaffected

To examine whether Epo-induced Stat5 phosphorylation is also affected *in vivo*, freshly isolated bone marrow cells from *wt* and *Fancg*$^{-/-}$ mice were stimulated with Epo after a brief (3h) factor deprivation. Epo-induced phosphorylation of Stat5 can not be detected by Western blot in a heterogeneous mixture of bone marrow cells. Therefore Epo-induced Stat5 DNA binding to the classical binding site β-casein was analyzed. Surprisingly, Stat5 activation in *Fancg*$^{-/-}$ cells was similarly efficient as in *wt* littermates (Figure 4A). In addition, induction of Stat5 target genes PIM1 and CIS as assessed by northern blot was also unaffected both in *Fancg*$^{-/-}$ (Figure 4B). This may suggest (*i*) a difference between erythroblasts derived from fetal liver and bone marrow or (*ii*) that reduced signaling efficiency may develop during cell culture.

Impaired Epo-signaling is restored by erythroid expansion at 3% oxygen

FA cells are hypersensitive to oxidative stress [45]. Notably, the atmospheric oxygen level (20%) during expansion of erythroblasts *in vitro* is cytotoxic particularly for mouse cells [46-47] and is significantly higher than physiological levels (~3%). Oxidative stress induces many of the DNA lesions with which cells have to cope continuously [48] and cells accumulate DNA damage more rapidly at 20% oxygen compared to 3% oxygen [47]. FA pathway-deficient cells are particularly sensitive to these culture conditions. Therefore we examined whether impaired Epo-induced phosphorylation of Stat5 was due to hyperoxic culture conditions and thus could originate from prolonged oxidative stress. Fetal livers from *Fanca*$^{-/-}$ mice and *wt* littermates were split and erythroblasts were expanded for 6 days at 20% and 3% oxygen. While Epo-induced Stat5 phosphorylation was impaired in cells expanded at atmospheric oxygen, Stat5-phosphorylation in response to Epo was not affected when progenitors were grown at 3% oxygen (Figure 4C). Thus, sensitivity to oxidative stress is involved in impaired signal transduction that develops during cell culture.

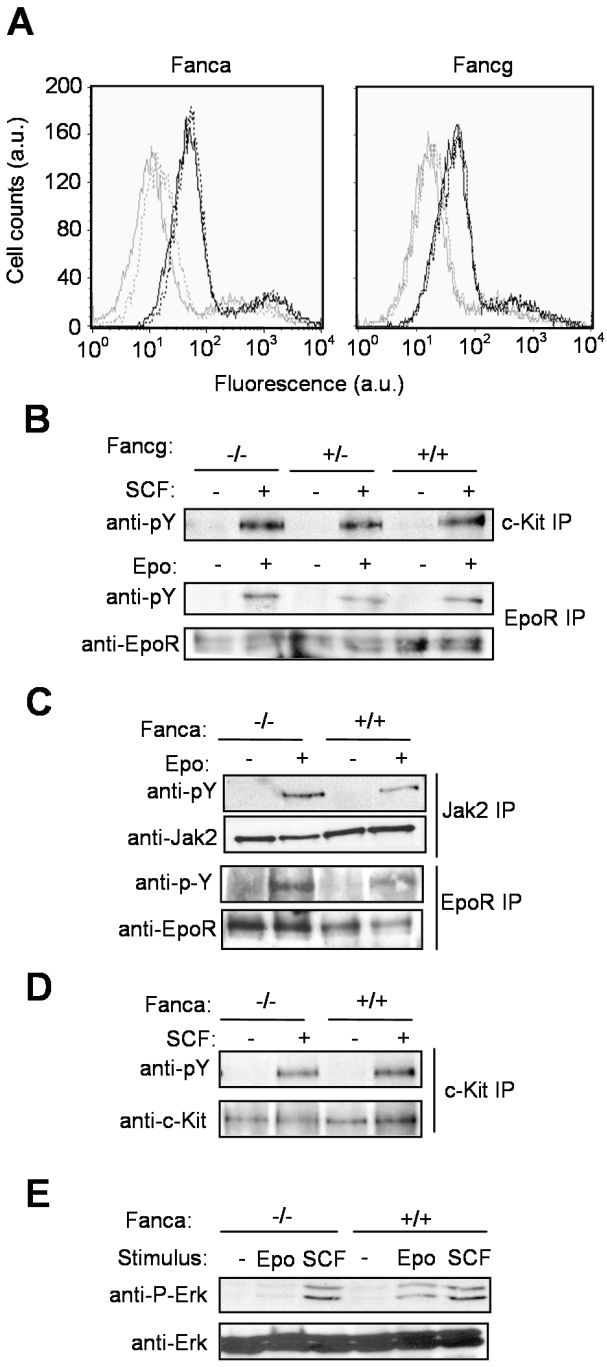

A
Fanca
Fancg
Cell counts (a.u.)
200
160
120
80
40
0
10^0 10^1 10^2 10^3 10^4
Fluorescence (a.u.)
B
Fancg: -/- +/- +/+
SCF: - + - + - +
anti-pY
c-Kit IP
Epo: - + - + - +
anti-pY
anti-EpoR
EpoR IP
C
Fanca: -/- +/+
Epo: - + - +
anti-pY
anti-Jak2
Jak2 IP
anti-p-Y
anti-EpoR
EpoR IP
D
Fanca: -/- +/+
SCF: - + - +
anti-pY
anti-c-Kit
c-Kit IP
E
Fanca: -/- +/+
Stimulus: - Epo SCF - Epo SCF
anti-P-Erk
anti-Erk

Figure 3: Ligand-induced phosphorylation of EpoR, Jak2 and c-Kit is not affected in Fanca$^{-/-}$ and Fancg$^{-/-}$ fetal erythroblasts.
(A) Expression of the EpoR on the surface of wt (dotted line), Fanca$^{-/-}$ (left panel, solid line) or Fancg$^{-/-}$ (right panel, solid line) erythroblasts was measured by flow cytometry, using biotinylated Epo. Black lines depict cells stained with biotinylated Epo, while the grey lines depict cells treated with biotinylated Epo in presence of 10 times excess of non-biotinylated Epo as a specificity control. B-E) wt (+/+), Fancg$^{-/-}$ (-/-) (B) or Fanca$^{-/-}$ (-/-) (C-E) fetal liver erythroblasts expanded for 6 days were factor deprived for 4h and stimulated with Epo (5U/ml; 10') or SCF (500ng/ml; 5') as indicated. Cells were lysed and the EpoR (B, C), Jak2 (C) and c-Kit (B, D) were immune-precipitated (IP). Immune-precipitates were analyzed on Western blots using the anti-phosphotyrosine antibody PY99 (anti-pY, upper panels) or the specific antibodies used in the IP as loading control (lower panels). E) whole cell lysates (WCL) of wt (+/+) or Fanca$^{-/-}$ (-/-) fetal liver derived erythroblasts were tested on Western blot for Erk phosphorylation.

Impaired Epo-signaling is a hallmark of defective ICL repair

Cells lacking the NER/ICL-repair enzyme Ercc1 are also extremely sensitive to oxidative stress [49-50]. *Ercc1$^{-/-}$* mice develop bone marrow hypoplasia at 3 weeks of age and have reduced expansion of erythroblasts similar to *Fancg$^{-/-}$* and *Fanca$^{-/-}$* cells [16]. The functions of the Fanconi pathway and Ercc1 converge at the ICL [51]. Therefore we investigated whether deficient EpoR signaling can more generally result from a deficiency in ICL repair. Erythroblasts were expanded from *Ercc1$^{-/-}$* fetal livers at atmospheric oxygen. Epo-induced Stat5 phosphorylation was significantly decreased in all expanded *Ercc1$^{-/-}$* progenitors compared to progenitors derived from *wt* littermates (Figure 5A). Surprisingly, *Xpa$^{-/-}$;Csb$^{m/m}$* mice, although having a premature aging phenotype and increased tumor risk, were unaffected in Epo-induced Stat5 and Erk phosphorylation (Figure 5B) and showed no expansion defect [49]. This indicated that defects in nucleotide excision repair *per se* do not lead to attenuation of Epo-signaling. In addition, Epo-induced signaling was also suppressed in *wt* erythroblasts treated with a low dose of the DNA crosslinking agent MMC (Figure 6A), but agents that induce bulky lesions (e.g. daunarubicin) did not affect Epo-induced Stat5 activation (data not shown). The data suggest that reduced EpoR signaling is not specific for defects in the Fanconi pathway, but it may be specific for defects in ICL repair.

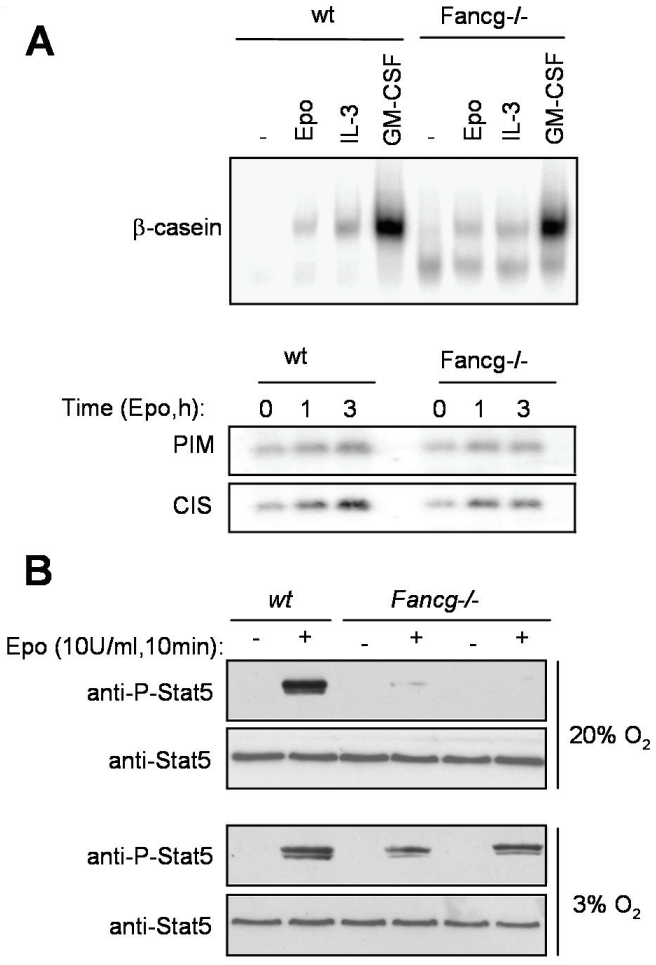


Figure 4: Impaired Epo signal transduction is induced by cell culture stress. *(**A**) Bone marrow was isolated from wt and Fancg$^{-/-}$ mice, incubated in serum- and factor-free medium for 3h and stimulated with Epo (5U/ml), IL-3 (supernatant) or GM-CSF (10ng/ml) for 10'. Nuclear extracts were generated and Stat5 binding to the β-casein probe by EMSA (electrophoretic mobility shift assay) was tested.* **B**) *Northern blots for Stat5 target genes PIM-1 and CIS performed on RNA isolated from fresh bone marrow samples of wt and Fancg$^{-/-}$ mice stimulated with Epo (5U/ml) for 0, 1 or 3 hours.* **C**) *Erythroblasts were expanded from wt or Fancg$^{-/-}$ fetal livers in 20% (upper panel) or 3% (lower panel) oxygen. After 6 days cells were factor deprived (4h) and stimulated with Epo (5U/ml). Lysates were examined on Western blots for the presence of Phospho-Stat5 (P-Stat5) and total Stat5.*

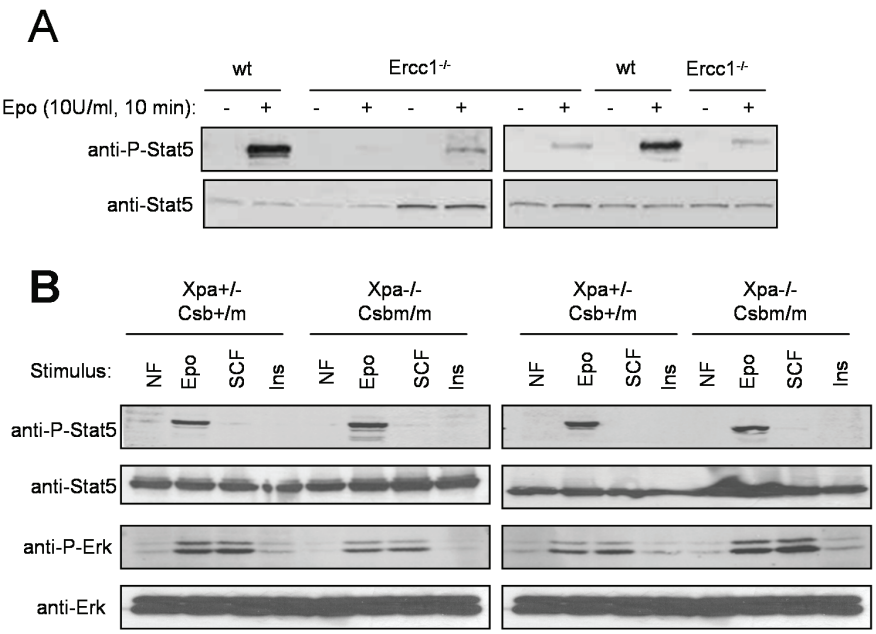


Figure 5: Ercc1$^{-/-}$ erythroblasts are also impaired in Epo-induced Stat5 phosphorylation.

*(**A**) Erythroblasts expanded separately from 2 wt and 4 Ercc1$^{-/-}$ fetal livers (5 days) were factor deprived (4h) and stimulated with Epo (5U/ml; 10'). Cell lysates were analyzed for the presence of phosphorylated Stat5 (P-Stat5) and total Stat5 (Stat5).* **B***) Aliquots from Xpa$^{+/-}$/Csb$^{+/m}$ and Xpa$^{-/-}$/Csb$^{m/m}$ erythroblast cultures at day 6 were starved (3h) and stimulated with Epo (10U/ml), SCF (100ng/ml), Insulin (Ins; 40ng/ml) or no factor (NF), lysed and whole cell lysates were subjected to SDS-PAGE and western blotting. Blots were stained using antibodies against phosphorylated Stat5 (P-Stat5), total Stat5 (Stat5), phosphorylated Erk (P-Erk) and total Erk (Erk). No defect in Epo or SCF signal transduction was observed.*

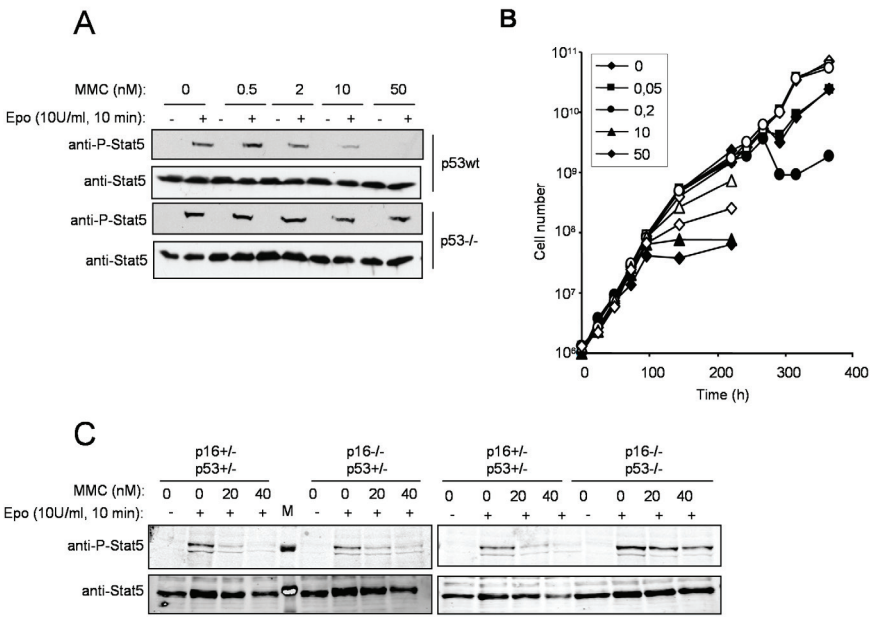


Figure 6: Genotoxic stress impaired Epo-induced Stat5 phosphorylation requires p53 but not p16.

*(**A**) Erythroblasts were expanded for 4 days from wt and p53$^{-/-}$ fetal livers in the continuous presence of MMC at different concentrations as indicated. Cells were factor deprived (4 h) and stimulated with Epo (5U/ml; 10'). Cell lysates were analyzed for the presence of phosphorylated Stat5 (P-Stat5) and total Stat5 (Stat5).* **B***) Erythroblasts derived from wt (closed symbols) and p53$^{-/-}$ (open symbols) fetal livers were cultured in serum free expansion medium containing Epo, SCF and dex in the presence of various concentrations of MMC as indicated (concentration in nM).* **C***) Erythroblasts from various p16/p53 heterozygous and knockout mice fetal liver culture were incubated with 0, 20 or 40 nM MMC for 24 h. Cells were then factor deprived (4h), stimulated with Epo (5U/ml; 10') and cell lysates were assayed by western blot for phosphorylation of Stat5 (P-Stat5) and total Stat5 (Stat5) on the same blot (M=protein marker).*

Impaired Epo signaling is rescued by loss of p53

Stalled replication forks result in phosphorylation and stabilization of p53, and in activation of the *Cdkn2a* locus that encodes $p19^{Arf}$ and $p16^{Ink4a}$, causing senescence or apoptosis [52-53]. To examine whether stalled replication forks interfere with the efficiency of signal transduction through a p53-dependent mechanism, erythroblasts expanded from *wt* and *p53*$^{-/-}$ fetal livers were exposed to increasing concentrations of MMC for 4 days and Epo-induced phosphorylation of Stat5 was examined. Addition of 2 to 50nM MMC reduced Epo-induced phosphorylation of Stat5 and Erk without affecting total Stat5 expression (Figure 6A). The same concentrations of MMC did not affect Epo-induced phosphorylation of Stat5 in absence of p53 (Figure 6A) and rendered the cells less sensitive to the growth inhibitory effect of MMC (Figure 6B). Indeed, p53 deficiency also rescued the reduced Epo-induced signaling observed in *Ercc1*$^{-/-}$ erythroblasts (Figure 7). Of note, a sub lethal dose of 2nM MMC during *wt* erythroblast expansion led to the same abrogation of expansion as observed for *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ after 10 days (compare Figure 1A, B to Figure 6B, 2nM MMC *wt* curve). Epo-induced phosphorylation of Stat5 in *Cdkn2a*$^{-/-}$ erythroblasts was still attenuated upon treatment with MMC ruling out a role for p16 in the signaling phenotype (Figure 6C). Together the data suggest that the observed signaling defect is part of a general senescence program induced upon specific DNA damage which is p53 but not p16 dependent.

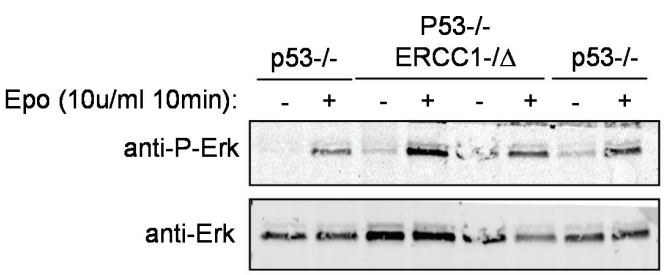


Figure 7: *The reduced Epo-induced signaling observed in Ercc1*$^{-/-}$ *erythroblasts is rescued by p53 deficiency. Erythroblasts were grown from two p53*$^{-/-}$ *and two p53/Ercc1-/delta knockout fetal livers for 6 days. Cells were factor deprived (4h), stimulated with Epo (10U/ml; 10') and cell lysates were assayed by western blot for phosphorylated Erk (P-Erk) and total Erk (Erk) on the same blot.*

DISCUSSION

One of the consistent and early symptoms of Fanconi anemia is bone marrow failure. *Fancc*$^{-/-}$*/Fancg*$^{-/-}$ mice develop bone marrow failure and leukemia [18], and *Fancc*$^{-/-}$ bone marrow cells grown at atmospheric oxygen for 4 days display reduced repopulation ability [21]. Consistent with these observations, *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts displayed a reduced *in vitro* expansion capacity and impaired Epo signaling that results in impaired Epo-controlled gene regulation. Importantly, the defect in Epo signaling is not observed in freshly isolated cells, but develops during *in vitro* cell culture at 20% oxygen. *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ progenitors retain their Epo-response when cultured at physiological oxygen levels (3%). Impaired Epo-induced phosphorylation of Stat5 was also observed in erythroblasts lacking the DNA repair enzyme Ercc1, in cells treated with sub lethal doses of MMC but not in *Xpa/Csb* double knockout cells and not in cells treated with genotoxic substances that generate bulky lesions (e.g.daunarubicin). Induction of the signaling defect by MMC required the presence of p53 but was independent of p16. Based on these findings, we suggest that reduced responsiveness to Epo in erythroblasts with a defective FA pathway is part of a general cellular response to stalled replication forks dependent on p53 and not due to a specific function of the FA pathway in Jak/Stat signal transduction.

Reduced EpoR signaling in Fanca$^{-/-}$ *and Fancg*$^{-/-}$ *erythroblasts*

We observed a reduction in Epo-induced phosphorylation of Stat5 and Erk1/2 in *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ erythroblasts expanded at atmospheric oxygen, paralleled by impaired regulation of Epo-target genes. In contrast, Epo-induced phosphorylation of Jak2 and the EpoR, as well as EpoR membrane expression was not impaired. This indicated that receptor activation *per se* is not affected, only the recruitment and/or phosphorylation of effectors downstream of the EpoR/Jak2 complex. SCF-induced phosphorylation of Erk and auto-phosphorylation of c-Kit is also not affected, suggesting that the signaling defect in hematopoietic FA knockout cells may be specific for Jak-dependent cytokine receptors. FA patients that develop bone marrow hypoplasia have high serumlevels of Epo [35-36], suggesting that erythroblasts in the bone marrow of FA patients fail to respond to Epo, when anemia becomes apparent.

Epo-induced Stat5 phosphorylation is also impaired in erythroblasts expanded from mice lacking the interstrand crosslink repair enzyme Ercc1, and in *wt* progenitors treated with a sublethal dose MMC, suggesting that the signaling defect is not specific for FA. Because the signaling defect does not occur in *Xpa*$^{-/-}$*Csba*$^{m/m}$ bone marrow it is not a general DNA damage effect. Instead it seems to be the result of replication stress. Mouse cells in culture are particularly sensitive to atmospheric oxygen, resulting in increased DNA damage [47]. The impaired DNA repair ability of cells defective in the FA-pathway will lead to increased chromosomal instability at 20% oxygen [54]. Thus, expansion of cells at hyperoxic conditions is likely to accelerate accumulation of DNA-damage and hence activation of p53 in comparison to *in vivo* conditions.

At present we do not know the mechanism that is causing the inefficient phosphorylation of Stat proteins and other signaling molecules in an EpoR but not SCF specific manner. This peculiar specificity rules out a general cellular response to genotoxic stress that interferes with signal transduction per se. The observation that SCF-induced signal transduction is not affected may bear importance because bone marrow failure in FA has a high risk of transformation into leukemia. Retained SCF-induced signals together with alterations in the signaling network during SIPS may be at the basis of cellular transformation leading to tumorigenesis [55 , 56].

Previously it was shown that Stat1 activation by IFNγ is impaired in *FANCC*$^{-/-}$ lymphoblastoid cell lines [27]. It was suggested that FANCC is directly involved in recruitment of Stat1 to the IFNγ-receptor [57]. Our data suggest that impaired Epo-induced Stat5 phosphorylation is a more general, p53-mediated response to replication stress, which could occur in all FA complementation groups as well as in other genomic instability syndromes or in *wt* cells exposed to DNA-damaging drugs.

Reduced expansion capacity of Fanca$^{-/-}$ *and Fancg*$^{-/-}$ *erythroblasts*

Fanca$^{-/-}$ or *Fancg*$^{-/-}$ erythroblasts are initially not affected in their expansion rate. Epo-levels were below the detection limit of 20U/l in both *Fanca*$^{-/-}$ and *Fancg*$^{-/-}$ mice, the number of erythrocytes and reticulocyte in blood was normal and comparable between *Fanca*$^{-/-}$, *Fancg*$^{-/-}$ and *wt* mice (reticulocytes: 2-5%; data not shown). Fetal liver size and weight was identical between *wt* and Fanconi-knockout embryo's (data not shown). Thus, there appears to be no defect in *in vivo* erythropoiesis in these mice. *In vitro*, however, the ability of the erythroblasts to undergo renewal divisions in response

to Epo, SCF and Dex is abrogated after circa 9-10 days, while *wt* cells can be expanded for more than two weeks before the cultures succumb to complete differentiation. Thus, the signaling defect clearly precedes inhibition of proliferation. Purification of *Fanca*$^{-/-}$ or *Fancg*$^{-/-}$ progenitors during this stage showed that mainly the population of large blastoid cells rapidly decreased without an increase in dead cells, suggesting enhanced differentiation. Enhanced differentiation at the expense of erythroblasts expansion was also observed for *wt* erythroblasts treated with a critical, sublethal dose of MMC, but did not occur in *p53*$^{-/-}$ erythroblasts.

Reduced expansion could be due to attenuated Epo-induced activation of downstream targets or it could be an independent cellular response to DNA damage or a combination of both. We previously demonstrated that loss of Btk decreases the efficiency of EpoR signaling, which lead to abrogation of expansion in favor of differentiation [41]. This suggests that expansion and differentiation require distinct Epo-signaling thresholds with lower Epo signaling resulting in enhanced differentiation. However, also increased p53 levels evoked by increasing DNA damage may abrogate the potential of erythroblasts to expand. p53 was shown to enhance differentiation and to abrogate proliferation of erythroblasts [58-59] and loss of *p53* is a crucial step in Friend virus-induced erythroleukemia [60]. Notably, p53 is increased in all tissues of *Fancc*$^{-/-}$ mice [61]. Lack of *p53* also abrogates the hypersensitivity of *Fancc*$^{-/-}$ cells for MMC, indicating that DNA damage-induced p53 is an important mechanism in the FA phenotype [61]. Interestingly, reduced phosphorylation of Stat5 induced by MMC, was rescued by *p53* deficiency, clearly suggesting a link between p53 and Epo-induced signaling. The gatekeeper p53 appears to play a major role in inducing premature senescence and aging, resulting in a reduction of the pool of stem- and progenitor cells [56, 62-63]. The data suggest that during hyperoxic culture erythroblasts lacking an intact FA pathway accumulate DNA damage and by consequence activate p53, which enhances expression of cell cycle inhibitory and differentiation promoting genes. If increased differentiation at the expense of renewal is due to impaired signaling and/or increased DNA damage due to cell culture, the expansion capacity is expected to be retained when *Fanca*$^{-/-}$ cells are cultured at 3% oxygen. However, the expansion of *wt* as well as *Fanca*$^{-/-}$ erythroblasts was decreased when cultured at 3% oxygen. This observation was surprising, since expansion of mouse embryo fibroblasts is much improved at 3% oxygen.

In conclusion, we found that long term erythroblast expansion and Epo signal transduction is dependent on the integrity of ICL repair pathways in a p53 dependent manner. This *in vitro* phenotype may be involved in the erythropoiesis defect and the insensitivity to Epo as observed in FA patients.

ACKNOWLEDGEMENTS

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6

Wnt-signaling in erythroblasts is controled by p53

Judith Verhagen-Oldenampsen, Sarah Duit, Pavel Vaclavik,
Nuno Andrade, Ernst W. Müllner, Johannes Nimpf
and Marieke von Lindern

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Abstract

Wnt/β-catenin signaling is intimately involved in maintenance and proliferation of stem- and progenitor cells. In contrast to Wnt/β-catenin signaling, activation of p53 induces senescence and abrogates renewal. Permanently proliferating cultures of committed mouse erythroblasts can be reproducibly established from p53-deficient fetal liver or bone marrow in serum-free medium supplemented with erythropoietin, stem cell factor and glucocorticoids. We observed that *p53*$^{-/-}$ erythroblasts have increased β-catenin levels and decreased spontaneous differentiation rates compared to respective wild-type erythroblasts. Expression of β-catenin protein levels inversely correlated with expression of the p53 target gene *Siah1* (seven in absentia homologue 1), a ubiquitine ligase known to control β-catenin protein stability. Proliferation of the established *p53*$^{-/-}$ erythroblast line I/11 was critically dependent on Wnt/β-catenin signaling. Our data suggest a mutual antagonistic effect of p53 and Wnt/β-catenin signaling on the balance of proliferation and maturation of committed erythroblasts.

INTRODUCTION

Lifelong production of the appropriate numbers of erythrocytes requires a balance between HSC maintenance and erythroid commitment, lineage expansion and survival in response to erythropoietin (Epo) [1]. In response to hypoxia, erythropoiesis will be promoted because enhanced levels of Epo increase survival of erythroblasts [2], as well as via enhanced transient amplification of erythroblasts, which involves stem cell factor (SCF) and glucocorticoids [3-4].

Erythroblasts can be expanded *in vitro* from mouse fetal liver or bone marrow in presence of Epo, SCF and glucocorticoids [5]. These cultures display limited self renewal for 10-15 cell doublings, and increasingly undergo spontaneous differentiation. Erythroblast cultures derived from p53 deficient mice display less spontaneous differentiation, which allows for the establishment of cultures with an apparent unlimited proliferation potential [6]. In addition, long-term p53 deficient cells retain a normal diploid karyotype and retain the ability to undergo terminal differentiation in presence of Epo and insulin [5-6]. To avoid genetic instability of p53 deficient cells under sub-optimal conditions [6], genetically stable cultures can be established from wildtype (wt) erythroblasts derived from embryonic stem cells (ESEPs [7]) or early fetal livers [8]. These long term cultures behave like their freshly isolated fetal liver derived *wt* counterparts with respect to cytokine/hormone requirement and terminal differentiation into enucleated erythrocytes, thereby producing cell numbers which are sufficient for complex biochemical studies [9-11].

The Wnt pathway is a highly conserved pathway which plays a crucial role in cell fate determination, survival, proliferation and movement in a wide variety of tissues [12]. Aberrant expression or function of Wnt signaling components is associated with several cancer types [13-16]. Wnt signaling cooperates with signals from several receptor tyrosine kinases and cytokine receptors to enable proliferation and survival as well as renewal of HSCs [17-21]. Multiple Wnts are expressed in hematopoietic tissues, raising the possibility of autocrine and paracrine activation of Wnt signalling [22]. Cultivation of HSCs in Wnt-conditioned media significantly stimulated proliferation and attenuated commitment/differentiation [18, 20]. In addition, the Wnt pathway was found to be constitutively activated in acute myeloid leukemia (AML) [23] and to be involved in the blast crisis of chronic myelogenous leukemia (CML) during progression to AML [24]. Moreover, the balance between HSC self-renewal and commitment seems to be regulated by Hmgb3 via canonical Wnt signalling [17, 25]. It remains

to be established, however, whether Wnts contribute to the renewal of hematopoietic progenitors at later stages of their development. This raised the question whether the Wnt pathway is also active in committed erythroblasts and whether it contributes to the transient amplification within the erythroid lineage.

Two independent intracellular signalling pathways have been identified, the Wnt/β-catenin (canonical) pathway [26] and the Wnt-Ca^{2+}/PCP (noncanonical) pathway [27]. Wnt signals are transduced through receptors of the frizzled (Fz) family [28] and coreceptor LRP5/6, which is a member of the low density lipoprotein receptor family [29-30]. In addition, Wnt factors can bind tyrosine kinase receptors such as Ror2 [31] or Ryk [32], which inhibits canonical signaling. Both Wnts and Fzs are members of large protein families, currently compromising at least 19 Wnts and 10 Fzs in the mouse [33]. Each Wnt can bind several Fz-receptors, but the specificity of Wnts for Fz-receptors is not yet fully understood. The key signal mediator in the canonical pathway is β-catenin, which is rapidly degraded in absence of Wnt signals. Wnt signaling stabilizes β-catenin, resulting in its translocation to the nucleus and association with members of the TCF/LEF family of transcription factors, which triggers transcription of target genes [26]. Expression and stability of β-catenin is regulated via two different pathways. The general pathway involves glycogen synthase kinase 3-β (GSK3-β) and the adenomatosis polyposis coli gene product, APC [26]. In the alternative pathway, a complex of Siah-1 (Seven-In-Absentia homologue-1), SIP (Siah-1-interacting protein SIP), Skp1 (S-phase kinase-associated protein 1) and Ebi binds to β-catenin to induce its ubiquitination and degradation [34-35]. Within this non-canonical pathway, the p53 controlled Siah-1 is the limiting factor [36].

Here we report that loss of p53 decreased Siah-1 expression, and increased β-catenin levels in fetal liver- and bone marrow derived erythroblasts, and in mouse embryonic fibroblasts. Downregulation of β-catenin inhibited proliferation and induced differentiation of p53-deficient erythroblasts. These results suggest a mutual antagonistic effect of the senescence promoting activity of p53 and Wnt induced “renewal signaling” on the balance between proliferation and differentiation.

MATERIALS AND METHODS

Cells and cell culture

Primary erythroblasts were isolated and expanded from fetal liver of E12.5 mouse embryos as described [5] using serum-free medium (Stem-Pro-34TM, Invitrogen), supplemented with 1U/ml human recombinant erythropoietin (kind gift of Ortho Biotech, Tilburg), 50ng/ml murine recombinant SCF (supernatant of producer cells), 10^{-6}M dexamethasone (Dex; Sigma) and 40ng/ml insulin-like growth factor 1 (IGF-I; Promega). Established erythroid lines (clone I/11 [6], R10, 2C4 [37], and ESEP [7]) were cultured under the same conditions. All erythroid cell types were expanded by daily partial medium changes and addition of fresh factors, keeping cell densities between 2-4 x10^6cell/ml. Cell number and size distribution were determined using an electronic cell counter (CASY-1, Schärfe-System). Proliferation kinetics and cumulative cell numbers were calculated as described [6]. To induce differentiation, cells were transferred to medium containing 10U/ml Epo, 10ng/ml insulin (ins; Actrapid HM), and 1mg/ml iron saturated human transferrin (Sigma).

Cytospins and hemoglobin assay

Cytospin preparations and staining with cytological dyes, and hemoglobin content was analyzed photometrically as described [38]. Results are the average of triplicate measurements after normalization for both cell number and volume.

Western Blot analysis

Erythroid cells were washed twice with ice-cold PBS, centrifuged and the pellet lysed in an isotonic buffer containing 10mM HEPES pH=7.5, 40mM KCl, 0.2% NP-40, 5% glycerol, 1mM DTT supplemented with proteinase inhibitor mix. Cell debris was removed by centrifugation for 5 min at 15.000 rpm at 4°C. Lysates were resuspended in 4x SDS loading buffer. Sample amounts to be loaded were normalized to both cell number and cell volume and subjected to Western Blot analysis as described earlier [6].

Antibodies used: anti-β-catenin monoclonal antibody (C19220, Transduction Laboratories), anti-ERK 1/2 (V1141, Promega), anti-HA (MMS-101R, Covance), a polyclonal anti-LRP6 antibody was raised in rabbit against the recombinant cytoplasmic tail of LRP6.

Proteins were either detected by fluorescently labeled secondary antibodies followed by detection using an Odyssey IR Imaging System (LI-COR Biosciences) or by probing with horseradish peroxidase-conjugated species specific antiserum (DAKO A/S, Glostrup,Denmark), followed by enhanced chemiluminescence reaction (Du-Pont, Boston, MA). Western blot results were quantified by integrating optical densities of bands using Gel-Pro Analyzer™ Software (Media Cybernatics, UK) and normalized to signals from respective loading controls.

cDNA preparation and PCR analysis

Total RNA was isolated using Tri ReagentÒ-RNA/DNA/Protein Isolation Reagent (Molecular Research Center, Inc.) and used for cDNA synthesis using SuperScript™ Reverse Transcriptase (Life Technologies, Inc.). PCR reactions were performed according to standard procedures: *Siah1b* mRNA expression was analyzed by quantitative PCR using Taqman Q-PCR as described [11]. Siah1b levels were normalized against expression of RNAse inhibitor. All other real-time PCR reactions were performed using the LightCycler® (Roche Diagnostics) in 20µl format using LightCycler DNA-Master SYBR Green I (Roche Diagnostics) and normalized against β-actin mRNA.

RNAi assays

Double-stranded siRNA oligomers were designed and manufactured by Invitrogen (Stealth Select RNAi). β-catenin siRNA sense: 5'-ccc aga aug ccg uuc gcc uuc auu a-3', antisense: 5'-uaa uga agg cga acg gca uuc ugg g-3'; non-functional siRNA sense: 5'-cca uug uuu gug cag uug cuu uau u-3', antisense: 5'-aau aaa gca acu gca caa aca aug g-3'. *Wt* and *p53*$^{-/-}$ fetal liver derived erythroblasts were transfected with siRNA using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Optimization of transfection conditions was done using a fluorescein-labeled double-stranded RNA (BLOCK-iT fluorescent oligo, Invitrogen).

RESULTS

Loss of p53 increases β-catenin levels

Because Wnt/β-catenin signaling enhances renewal of stem cells or multipotent hematopoietic progenitors [19], we compared β-catenin levels in long-term cultures of *p53*$^{-/-}$ versus *wt* fetal liver-derived erythroblasts. The *p53*$^{-/-}$ clone I/11 displayed significantly elevated levels of cytoplasmic β-catenin in comparison to *wt* erythroblasts (Figure 1A). Moreover, a considerable fraction of β-catenin in I/11 cells localized to the nucleus, consistent with a function in transcriptional signaling (Figure 1B). To analyze whether these high levels of β-catenin were a direct consequence of p53 loss or rather a clonal feature of I/11 cells, β-catenin expression was measured in independently obtained long-term cultures of *p53*$^{-/-}$ erythroblasts derived fetal livers (FL) or bone marrow (BM), versus *p53*$^{+/+}$ embryonic stem cell-derived erythroid progenitors (ESEPs; [7, 37]) (Figure 1C). Both early (7 days) and long-term (up to 60 days) p53-competent ESEP cultures showed low levels of β-catenin, whereas all *p53*$^{-/-}$ established erythroblast cell lines exhibited high β-catenin expression, independent of clonal background (72a, R10, both from fetal liver) or tissue (2C4, bone marrow) of origin (Figure 1C).

To investigate whether β-catenin levels were already elevated in p53-deficient primary cultures or in established cell lines only, β-catenin expression was compared between freshly isolated cultures of *p53*$^{-/-}$ and *wt* fetal liver-derived erythroblasts. These cells were derived either from *p53*$^{-/-}$ or *wt* animals, or from mice lacking the nucleotide excision repair enzyme Ercc1. The cross-link repair defect of *Ercc1*$^{-/-}$ erythroblasts results in p53-dependent reduced proliferation and enhanced differentiation (manuscript in preparation). Compared to *wt* erythroblasts, β-catenin expression was enhanced in primary erythroblast cultures derived from *p53*$^{-/-}$ fetal livers, whereas it was undetectable in erythroblast cultures (two independent lines) derived from *Ercc1*$^{-/-}$ mice.

Analysis of MEFs derived from either *p53*$^{-/-}$ or *wt* animals suggested that the observed upregulation of β-catenin was a common consequence of loss of p53 (Figure 1E) rather than a senescence-related event during prolonged cultivation.

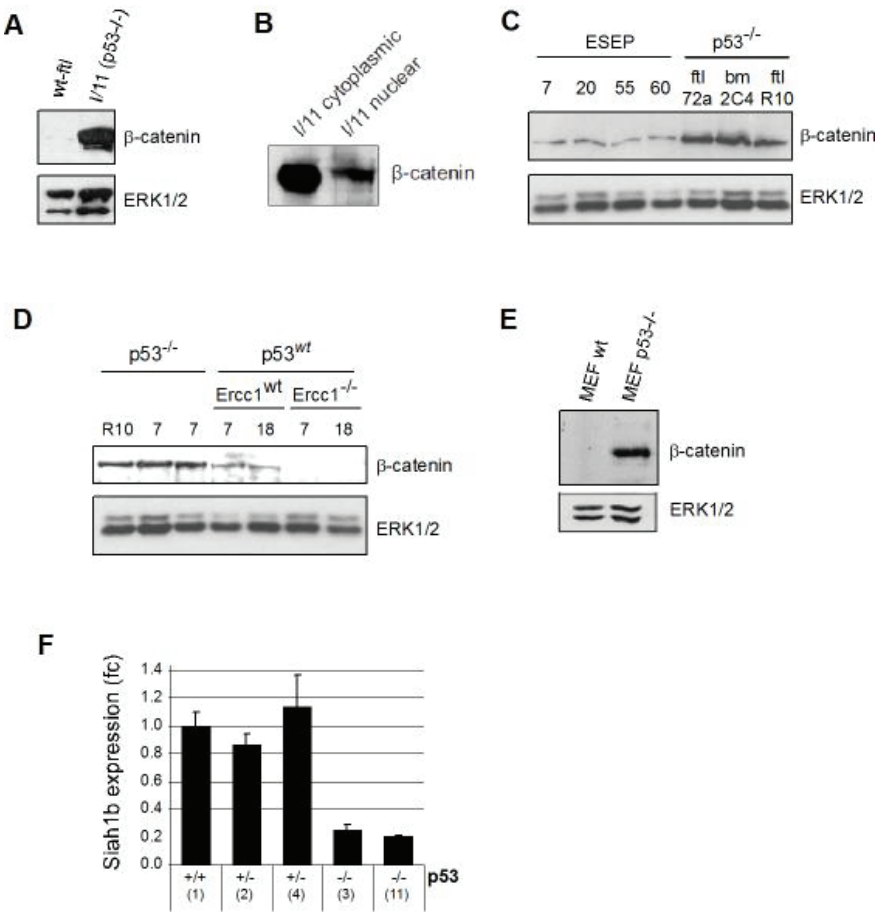

Figure 1: β-catenin protein levels are increased by p53-deficiency.
*A-F. Cell lysates were separated by SDS-PAGE and immunoblotted using antibodies specific for β-catenin or ERK1/2 (loading control). (***A***) Cytoplasmic extract of primary wt fetal liver-derived erythroblasts (wt-ftl) or the p53$^{-/-}$ erythroid cell clone l/11. (***B***) Cytoplasmic and nuclear β-catenin levels in the p53$^{-/-}$ erythroid cell clone l/11. (***C***) Wt embryonic-stem-cell-derived erythroblasts (ESEP; numbers indicate days in culture) and different established cultures of p53$^{-/-}$ erythroblasts (ftl 72a, R10: fetal-liver-derived erythroblasts; bm 2C4, bone-marrow-derived erythroblasts). (***D***) Erythroblasts freshly expanded from fetal livers of p53$^{-/-}$ mice (p53$^{-/-}$; numbers indicate days the cells were kept in culture; primary wt (p53^{wt}/Ercc1^{wt}) or Ercc1-deficient erythroblasts (p53^{wt}/Ercc1$^{-/-}$). (***E***) wt or p53$^{-/-}$ mouse embryo fibroblasts (MEF). (***F***) Siah1b mRNA expression was quantified in erythroblasts cultivated for 7 days from wt, p53$^{+/-}$ and p53$^{-/-}$ fetal livers. Numbers identify littermates. Values are the average of at least 7 independent experiments; error bars indicate standard error of mean (SEM).*

Expression of β-catenin is mainly regulated by ubiquitination and proteosomal degradation. Two distinct ubiquitin ligases are involved: APC and Siah1, whereby Siah1 expression is controlled by p53 [36]. We measured the expression of Siah1b in erythroblasts cultured for 7 days from *p53*$^{-/-}$, *p53*$^{+/-}$, or *wt* fetal livers of embryos from the same litter by quantitative PCR (qPCR). Whereas *Siah1b* mRNA levels were similar in *p53*$^{+/-}$ and *wt* cultures, expression was 5-fold lower in *p53*$^{-/-}$ cultures (Figure 1F). Expression of Siah1a was not detectable in any of the samples analyzed.

Together, these experiments demonstrated a strict inverse correlation between β-catenin levels and p53 expression that was not restricted to the erythroid lineage but also observed in primary fibroblasts. Further, the lack of p53 and increased expression of β-catenin were associated with low levels of the p53 target gene Siah1b, that is known to control β-catenin degradation.

	Self-renewal		Differentiation	
Wnt genes	wilt-type	p53-/-	wilt-type	p53-/-
Wnt1, 2	-	-	-	-
Wnt2b	-	-	++	-
Wnt3, 3a, 4	-	-	-	-
Wnt5a	+	-	-	-
Wnt5b, 6, 7a, 7b, 8a, 8b, 9a, 9b, 10a	-	-	-	-
Wnt10b	-	++	-	-
Wnt11	+	-	-	-
Wnt16	-	-	-	-
Frizzled genes				
Fzd1, 8	-	-	-	-
Fzd2	++	-	-	-
Fzd3	++	+	+	-
Fzd4	++	-	+	-
Fzd5	+	-	+	-
Fzd6	++	-	++	-
Fzd7	++	+	+	+
Fzd9	++	+	+	+
Fzd10	++	-	-	-
Kremen genes				
Krm1	++	++	+	+
Krm2	-	-	-	-

Table 1: Expression of components of the Wnt signaling pathway in p53-deficient (l/11) and wild-type erythroid progenitors during self-renewal and differentiation.

Expression of Wnts, Frizzled (Fzd) receptors and Kremen (Km) transcripts was analyzed by qPCR. Symbols used: -, no expression; +, weak expression; ++, strong expression.

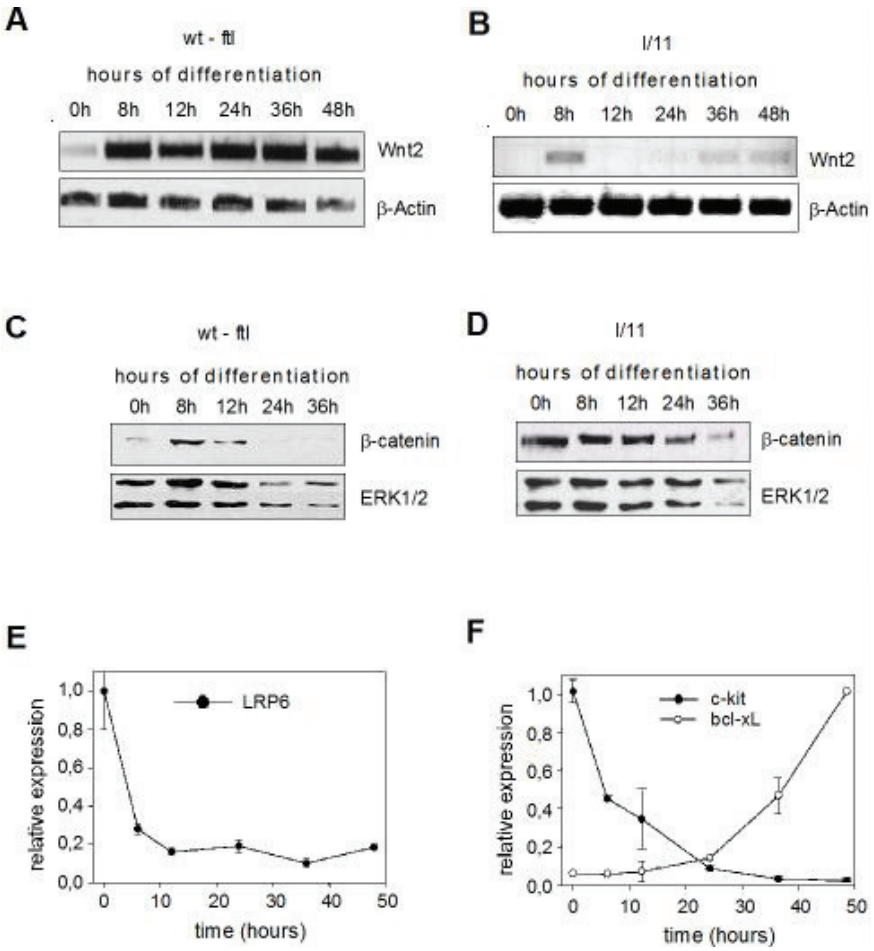

Figure 2: Expression of Wnt2b, β-catenin, and LRP6 during differentiation of wt versus p53-deficient mouse erythroblasts.

*mRNAs prepared from wt fetal liver erythroblasts (wt-ftl) (***A***) or the p53$^{-/-}$ erythroid cell clone l/11 (***B***) at 0, 8, 12, 24, 36, and 48 hours after induction of differentiation, respectively, were used for qPCR using primer pairs specific for Wnt2b and β-actin (as constitutive expression control) followed by gel electrophoresis. Cell lysates from wt erythroblasts (wt-ftl) (***C***) or p53$^{-/-}$ l/11 cells (***D***) were prepared at different stages of differentiation, separated by SDS-PAGE, immunoblotted, and detected using antibodies specific for β-catenin or ERK1/2 (loading control for normalization). Expression levels of LRP6 (***E***), c-Kit, and Bcl-X$_L$ (***F***), the latter two as controls for known changes in expression patterns during erythroid differentiation in l/11 cells during differentiation measured by qPCR. mRNA was prepared from cells harvested at 0, 6, 12, 24, 36, and 48 hours after differentiation induction. Data normalized to β-actin mRNA. Error bars: SEM, n=3.*

Expression of Wnt/β-catenin signaling components in erythroblasts

Although reduced *Siah1b* expression can increase β-catenin levels, the pathway still requires activation by Wnt ligand. Therefore we compared expression of crucial components of the Wnt signaling pathway in $p53^{+/+}$ versus $p53^{-/-}$ erythroblasts during self-renewal and differentiation.

Using RT-PCR we detected significant amounts of 7 of the 10 known *Frizzled* genes (*Fz2, 3, 4, 6, 7, 9, and 10*) in *wt* and 53-deficient erythroblasts (Table 1). Most *Fz* mRNAs became less abundant during differentiation. Expression analysis for the 19 mouse *Wnt* mRNAs indicated that *wt* erythroblasts cultured under renewal conditions only expressed low levels of *Wnt5a* and *Wnt11* mRNAs (Table 1). Surprisingly, Wnt2b expression, so far described for its proliferation-promoting role in kidney, eye and liver development [39-41], increased massively during differentiation (Figure 2A,B). All other Wnt transcripts remained undetectable in terminally maturing erythroid cells. Only $p53^{-/-}$ I/11 cell clones expressed high levels of *Wnt10b* mRNA, which became downregulated during differentiation (Table 1). Most likely, this clone-specific genetic event occurred during the establishment of the I/11 cell line.

Besides Fz proteins, Wnt signaling also requires LRP5 and/or LRP6 (LDL-receptor related protein) co-receptors. *LRP6*, but not *LRP5* mRNA was expressed in proliferating erythroblasts, but became rapidly down-regulated as the cells started to differentiate (Figure 2B). As a control for differentiation efficiency, *c-Kit* mRNA abundance (SCF receptor; a marker for immature hematopoietic cells) and transcript levels of the anti-apoptotic gene *Bcl-Xl*, were used as surrogate markers, showing the expected down- and upregulation pattern, respectively [9-10, 42] (Figure 2F).

Analysis of β-catenin expression during differentiation of erythroblasts showed a transient upregulated at 8 hours after differentiation induction and decreased to undetectable levels at 24 hours (Figure 2B). This time window of elevated β-catenin expression can be explained by the upregulation of Wnt2b and concurrent downregulation of Lrp6.

Finally, also the expression of *Kremen1* and *Kremen2* mRNAs (receptors for Dickkopf-1; negatively regulating canonical Wnt signaling [43]) were analyzed by qPCR. *Kremen1* mRNA was expressed at comparable levels during self-renewal as well as differentiation (Table 1).

In conclusion, primary erythroblasts proliferating *in vitro* express multiple Fz receptors, low levels of Wnt5a and Wnt11 ligands. Wnt2b was specifically expressed during differentiation. Its signaling potential, however, appears limited due to the rapid downregulation of LRP6.

Down-regulation of β-catenin by RNAi in p53-deficient erythroblasts triggers apoptosis

To investigate whether activation of the canonical Wnt-pathway contributes to the expansion capacity of the I/11 erythroblastic cell line, β-catenin expression was down-regulated using RNA interference (RNAi). A β-catenin-specific shRNA expressed from the retroviral vector pMSCV, but not the control shRNA downregulated ß-catenin in mouse L-cell fibroblasts expressing Wnt3A (Figure 3A, insert).

Next the RNAi vectors were introduced into *p53*$^{-/-}$ I/11 cells. Control cells proliferated normally (Figure 3A) and displayed only few dying and/or differentiating cells, as deduced from size profiles and cytospins (Figure 3B,C). In contrast, cells transduced with the β-catenin specific RNAi vector proliferated slower and displayed a high proportion of apoptotic or abortively differentiated cells (Figure 3B,C). Increased cell death of the β-catenin knockdown cells was confirmed by propidium iodide staining plus flow cytometry (Figure 3D) and TUNEL-assays (Figure 3E), revealing a significantly higher proportion of apoptosis in comparison to cells expressing the control vector. β-catenin levels were massively reduced by the specific shRNA (data not shown). These data indicate that up-regulation of β-catenin is required for long-term expansion of *p53*$^{-/-}$ I/11 erythroid cells.

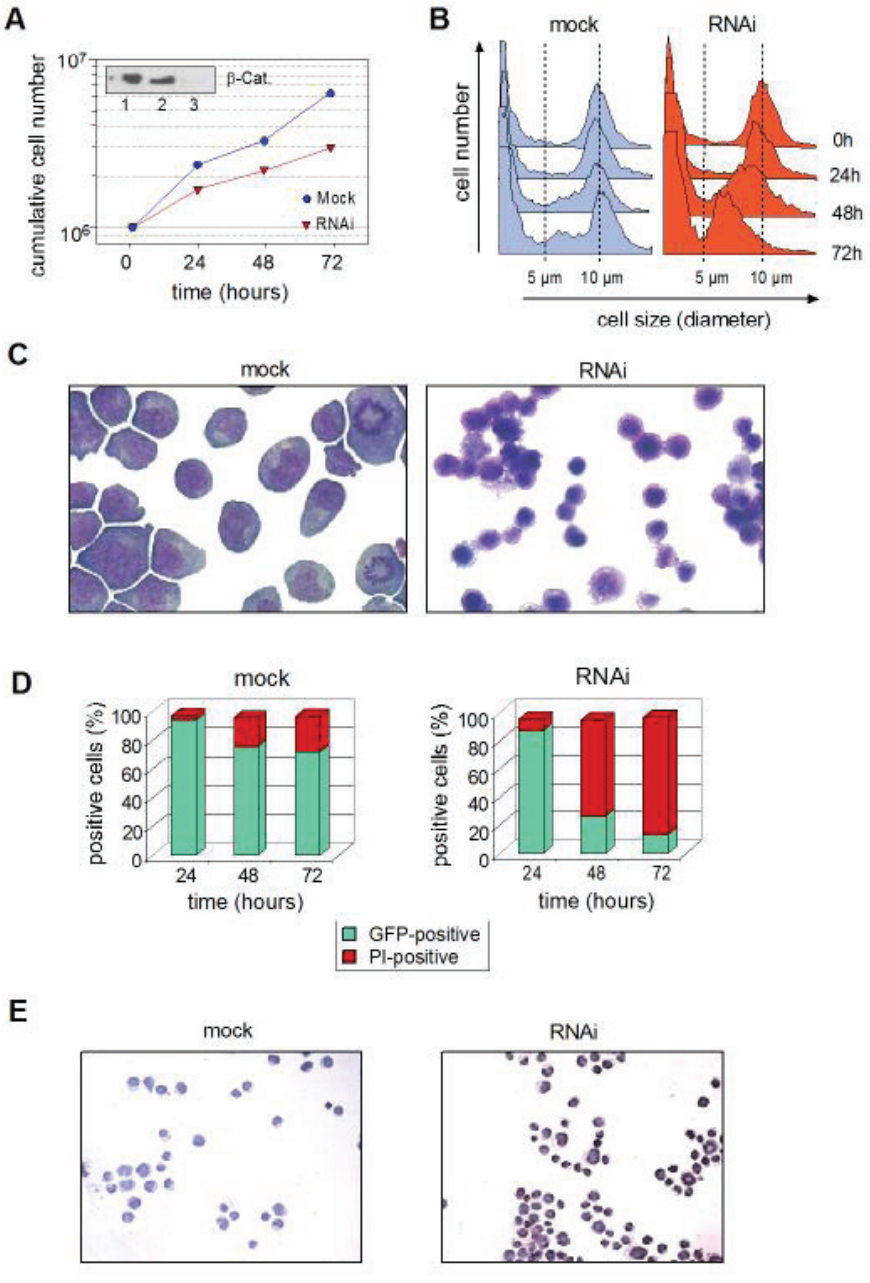
A
cumulative cell number
10^7
10^6
β-Cat.
1 2 3
Mock
RNAi
0 24 48 72
time (hours)
B
mock
RNAi
cell number
0h
24h
48h
72h
5 µm 10 µm 5 µm 10 µm
cell size (diameter)
C
mock
RNAi
D
mock
RNAi
positive cells (%)
100 80 60 40 20 0
24 48 72
time (hours)
GFP-positive
PI-positive
E
mock
RNAi

Figure 3: Down-regulation of β-catenin in p53-deficient erythroblasts by RNAi. *Insert in panel* **A***: β-catenin protein levels in L-Wnt3A cells infected with empty vector (lane 1) or infected with a pMSCV-based retrovirus bearing either a mismatched β-catenin RNAi construct (lane 2) or a functional β-catenin RNAi construct (lane 3), respectively. Cumulative cell numbers (***A***) and cell size distributions (***B***) of p53-deficient erythroblasts (I/11) infected with either a mismatched β-catenin RNAi construct (mock) or a functional β-catenin RNAi construct (RNAi). Cells were cultured under renewal conditions and analyzed every 24 hours. Note impaired proliferation (A) and gradual reduction of cell size in cells expressing functional β-catenin RNAi (B). (***C***) Cytospin preparations from aliquots of the above cultures 72 hours after transduction, were stained with histological dyes plus neutral benzidine (to reveal hemoglobin). (***D***) The proportion of propidium iodide positive erythroblasts was determined by flow cytometry in samples taken 24, 48 and 72 hours after infection. (***E***) After staining with haematoxylin (blue), apoptotic cells (brown/black) infected with empty vector (left panel) or β-catenin RNAi (right panel) were detected by TUNEL assay on cytospins.*

DISCUSSION

The Wnt/β-catenin signaling pathway is critically involved in expansion of various types of stem and progenitor cells, while activation of p53 restricts proliferation. Lack of p53 reduces spontaneous differentiation in fetal liver-derived erythroid cultures and allows for establishment of cultures with unlimited self-renewal that remain fully dependent on cytokines and hormones for proliferation and differentiation [44-45]. Although loss of p53 is thought to enable establishment of cell lines by allowing genetic instability, we previously showed that *p53*$^{-/-}$ erythroid cell lines, when properly treated, remain diploid and fully competent to differentiate into hemoglobinized, enucleated erythrocytes [6, 10]. Here, we demonstrate that loss of p53 contributes to enhanced activity of the Wnt/β-catenin pathway, which correlated with reduced expression of the p53 target gene *Siah1b*, encoding an E3-ligase for β-catenin. Our findings implicate that Wnt/β-catenin signaling does not only maintain renewal in stem cells, but also in fully committed progenitors and they suggest mutual antagonistic effects between the “senescence factor” p53 and the “renewal pathway” induced by Wnt.

Loss of p53 is associated with upregulation of β-catenin in erythroblasts

Primary erythroblasts of different origin undergo renewal divisions in response to SCF, Epo and glucocorticoids, [6-7, 10, 46-47]. Moreover, all primary *wt* erythroblasts, including ESEPs [7] and avian erythroblasts [48], undergo considerable spontaneous differentiation, even under optimal renewal conditions. In primary *p53*$^{-/-}$ erythroblasts, however, spontaneous differentiation is strongly reduced and even absent in established *p53*$^{-/-}$ cultures. Interestingly, all *p53*$^{-/-}$ cells tested, accumulated increased amounts of β-catenin whereas *wt* erythroblasts had low β-catenin levels, even when they were able to undergo long-term renewal (ESEP). This phenotype was not restricted to cells of erythroid origin but was also observed in MEFs. Erythroblasts deficient for the DNA cross-link repair gene *Ercc1* (*Ercc1*$^{-/-}$) undergo enhanced differentiation. Although p53 levels are too low to be detected, this process is p53-dependent as loss of p53 restores expansion of *Ercc1*$^{-/-}$ erythroblasts (Chapter 4). This could explain why *Ercc1*-deficient cells were completely devoid of β-catenin.

Siah-1b, the mammalian homologue of *Drosophila* Seven In Absentia, is a p53 target gene [34]. Siah-1 recruits E3 ubiquitin ligases and results in GSK3-independent degradation of β-catenin [34, 49]. Indeed, low Siah1b levels in freshly isolated primary erythroblasts from individual mouse embryos correlated with loss of p53 (Figure 1E). Taken together, our observations indicate a general mechanism for the inverse relationship between p53 activity and β-catenin levels.

The correlation between lack of p53 and enhanced β-catenin levels also sheds new light on the role of p53 in murine erythroleukemia induced by Spleen focus forming virus (SFFV) or Friend virus. The SFFV-encoded glycoprotein gp55 associates with the Epo-receptor, resulting in Epo-independent proliferation and polycythemia. Transformation to leukemia is critically dependent on activation of the transcription factors PU.1/Spi1 or Fli-1, combined with loss of p53 [50]. Loss of p53 was assumed to induce mutations that cooperate with PU.1 or Fli-1, but other studies indicate that it is required to protect cells from apoptosis and promote renewal of leukemic cells [51-52]. Our studies suggest that stimulation of Wnt/β-catenin signaling may be one of the effects resulting from loss of p53 in SFFV-induced erythroleukemia.

Recently, the Wnt pathway has also been implicated in human leukemia (reviewed in [53]). A large number of AML samples express increased levels of β-catenin and forced expression of β-catenin in hematopoietic progenitors impairs myelomonocytic differentiation [54]. Furthermore, transition of CML to blast crisis was shown to involve nuclear localization of β-catenin [55], while the *Frat*-oncogenes, activated upon retroviral insertion, induce T-cell leukemia by inactivation of glycogen synthase kinase 3 and β-catenin stabilization [56]. Finally, up-regulation of the Wnt coreceptor RYK and ß-catenin itself was found in several cases of myelodysplastic syndrome /AML with poor prognosis [57].

Autocrine Wnt/β-catenin signaling

Analysis of Wnt expression in early and established cultures of *wt* and *p53*$^{-/-}$ erythroblasts, indicated that low levels of several Wnt factors are detectable in all cultures. A high expression of Wnt10b was observed in established I/11 cells. Inhibition of ß-catenin expression in I/11 erythroblasts by RNA interference abrogated renewal, suggesting that a Wnt10b autocrine loop is required for sustained proliferation of I/11 cells. The observation that I/11 cells die instead of differentiate upon the RNAi treatment may be due to “conflicting instructions” given by renewal factors in the sudden absence of Wnt signaling. The other *p53*$^{-/-}$ cell lines, however, were established independently of significant expression of Wnt10b or any other Wnt. This difference between established cultures is not surprising, because cultures of p53-deficient erythroblasts go through a brief period in which proliferation and spontaneous differentiation balance each other before spontaneous differentiation decreases and proliferation increases. In I/11 erythroblasts the events leading to establishment of a permanently proliferating cell line apparently involved up-regulation of Wnt10b, other cultures may have been selected on the basis of distinct additional events.

Is the Wnt pathway required for in vivo and in vitro erythropoiesis?

The broad spectrum of Frizzled receptors (*Fz2, 3, 4, 5, 6, 7, 9*, and *10*) in *wt* erythroblasts suggests that the cells can respond to a variety of Wnts. We do not have evidence that *wt* erythroblasts produce Wnts enhancing their renewal. Instead, the cells may respond to paracrine stimulation by Wnts from stromal cells in the bone marrow or the spleen [18, 20, 58].

Interestingly, Wnt2 was absent in renewing *wt* fetal liver erythroblasts but strongly upregulated as soon as 8 hours after differentiation induction. Simultaneously the Fz-co-receptor LRP6 was down-regulated, which may explain the short time window in which Wnt2 could drive β-catenin signaling. Possibly this transient Wnt2 signal causes the transient increase in proliferation rate during the early "differentiation divisions" observed with chicken and mouse erythroblasts [6, 59].

The Wnt/β-catenin/TCF4 axis is crucial for proliferation of colon crypt stem cells and perturbed activation of TCF4 is a critical step in tumor development [60] and leukemia (reviewed in [53]). Recent studies showed that Wnt also promotes self-renewal of hematopoietic stem cells [18-19, 21] where it has been implicated in the proper balance between self-renewal and differentiation [17, 25]. Ablation of β-catenin in the hematopoietic system, however, did not show major defects [61]. Alternatively, enhanced β-catenin may only be required to counteract stress conditions, such as oxidative stress *in vitro* due to culture at atmospheric oxygen [62]. It is also possible that the effects of endogenous Wnt on stem cell renewal may only become apparent when the stem cell pool is challenged. Thus, in erythropoiesis, the requirement for Wnt/β-catenin signaling *in vivo* may become important when maximum expansion of the erythroid compartment is needed, i.e. under prolonged hematopoietic stress [46, 63]. Similar situations have been observed for mice expressing glucocorticoid- and Epo receptor mutants or deficient in STAT5, that show little or no hematopoietic defects *in vivo* (unstressed), whereas corresponding fetal liver-derived erythroblasts cultured *in vitro* exhibit decreased renewal and enhanced premature differentiation [47].

Stress erythropoiesis, in the mouse, occurs specifically in the spleen and not in the bone marrow. Expression of BMP4 and sonic hedgehog in spleen are important [64-65]. It would be interesting, however, to compare Wnt expression by stromal cells in mouse bone marrow and spleen.

In conclusion, several Wnts may act on erythroid progenitors. Stabilization of β-catenin is necessary but not sufficient to induce erythroblast renewal. Wnts cannot substitute for the renewal factors Epo, SCF or glucocorticoids. Instead, constitutive expression of Wnt5a allows long-term proliferation of *wt* erythroblasts, as previously observed for p53-deficient cells. Because p53-deficient cells express high levels of β-catenin, we postulate that Wnt/β-catenin signaling and p53 activation are mutually antagonistic. To understand the implications of this mutual antagonism under various conditions is the next challenge.

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7

Summary and Discussion

SUMMARY AND DISCUSSION

Summary

During the entire life time of an organism, its DNA is exposed to conditions that modify nucleotides or break the DNA strands. Defective repair of DNA damage causes mutations that may lead to cancer. The various tumour suppressor systems block survival and-or proliferation of mutated cells. However, protection from cancer causes aging of cells and tissues, and of the entire organism. The balance between protection and aging results in an increasing cancer incidence with age [1].

Much has been learned from syndromes linked to defective DNA repair. Yet little is understood as to why distinct DNA repair defects are linked to specific disease phenotypes [2]. The DNA damage response (DDR) consists of a complex network of proteins that are responsible for damage recognition, signal transduction, cell cycle control and DNA repair [3]. This network function is balanced between aging and cancer development [4]. Organismal aging and cancer development are tightly regulated by the expression level of several DDR proteins (such as ATR reviewed in [5]) as well as by the damage load [6].

Our blood contains a variety of cells with a relatively short lifetime and maintenance of blood levels requires the continuous generation of blood cells from the hematopoietic stem- and progenitor cell (HSPC) compartment in the bone marrow. By consequence, the hematopoietic system is very sensitive to DNA damaging drugs. The cytotoxic anticancer drugs used for most kinds of cancer have several adverse effects on hematopoiesis. For example, long term treatment with high dose alkylating agents may lead to progressive depletion of HSPCs and subsequent bone marrow hypoplasia. This hypoplastic environment can drive the remaining HSCs into proliferation while mutations caused by DNA damage result in a high risk of leukemic development.

The aim of this thesis was to elucidate the molecular mechanism of bone marrow failure and the subsequent leukemogenesis as seen in bone marrow failure syndromes associated with age or with persistent DNA damage. For this we used *Ercc1* deficient mice. Ercc1 is an endonuclease which is involved in the excision of nucleotides during global and transcription coupled nucleotide excision repair (NER) and it also functions in excision of interstrand crosslinks (ICLs) at the replication fork. It has previously been shown that the number of hematopoietic stem cells was reduced in the bone marrow of *Ercc1*$^{-/-}$ mice and that the capacity of BM progenitors to generate colonies was severely impaired [7].

In this study we first analyzed whether the severe progeria (overall aging) of the mice, which is associated with the defective NER, contributes to their bone marrow defect or whether it is specifically due to defective ICL repair at the replication fork (**Chapter 2**). Comparison with *Xpa*$^{-/-}$*Csb*$^{-/-}$ mice that lack NER but have intact ICL repair revealed that the severe progeria in *Ercc1*$^{-/-}$ mice does not cause aging of the BM compartment.

The alternative explanation, that HSPCs are particularly sensitive to defects in ICL repair and stalled replication forks was supported by the fact that the quiescent stem cells of the HSPC population are relatively less affected once the mice become adult. Conditional knockout mice as well as hematopoietic repopulation studies in which *Ercc1*-defective BM was transplanted into irradiated hosts indicated progressive loss of *Ercc1*-deficient cells. This is in line with the hypothesis that proliferation is a disadvantage for cells with decreased DNA repair capacity (**Chapter 3**).

Long term hematopoiesis could not be studied in *Ercc1*$^{-/-}$ mice due the fact that they die at three weeks of age. Mice with one hypomorph allele (*Ercc1*$^{-/d}$) live for ~20 weeks and mice with two hypomorph alleles (*Ercc1*$^{d/d}$) live for > 6 months. This difference in lifespan must be due to a dosage effect of their residual Ercc1 activity, and thus we next analyzed how small differences in Ercc1 activity affected parameters of progeria and hematopoiesis (**Chapter 3**). Although *Ercc1*$^{-/d}$ as well as *Ercc1*$^{d/d}$ mice liver significantly longer than *Ercc1* full knockout mice, they are still 50% smaller than their wildtype littermates. They also displayed signs of polyploidy in the liver which indicates that they age prematurely, however liver polyploidy decreased when the level of Ercc1 activity increased. The fraction of stem cells (LSK; lin-, Sca+, cKit+ cells) in the total BM of 3 and 10 week old Ercc1-deficient animals correlated to Ercc1 levels. Within the LSK compartment we detected a shift towards the more primitive cells (LT-HSC & ST-HSC) at the expense of lower number of mulitpotent progenitors in all Ercc1 deficient animals, which is associated with the largely quiescent state of primitive stem cells and the actively cycling state of MPP. Within the progenitor compartment there is a dramatic decrease of myeloid progenitors while lymphoid progenitors are not affected. Although low levels of Ercc1 increased the numbers of progenitors, these cells still failed to form BFU-E or CFU-G. Only the number of CFU-GM was slightly enhanced in *Ercc1*$^{d/d}$ mice, but *wt* numbers were never reached. The fact that a low Ercc1 dose prolongs the lifespan of Ercc1 deficient mice while hematopoiesis is still severely affected renders mice with a hypomorphic *Ercc1* allele suitable as a leukemogenesis model.

Progeria due to suppression of the hormonal growth axes has been linked to sustained DNA damage and defective TC-NER. As we established that the hematopoietic defect is independent of progeria and NER (**Chapter 2)**, we next examined which tumor suppressor genes were responsible for BM aging (**Chapter 4**). Expression of both the p53 target genes *Puma* and *Noxa*, and the genes encoded by the *Cdkn2a* locus, $p16^{Ink4A}$ and $p19^{Arf}$ was increased in *Ercc1* deficient bone marrow cells compared to their *wildtype* littermates. Deletion of either the *Trp53* or *Cdkn2a* locus in *Ercc1*-deficient mice revealed that p53 but not p16 or p19 contribute to loss of the HSPC compartment upon defective ICL repair. Intriguingly, loss of p53 enhanced the proliferative capacity of hematopoietic progenitors, but aggravated segmental progeria and liver polyploidy in *Ercc1*-deficient mice. Loss of p53 did not alter the primary molecular response to stalled replication forks, which is the formation of phosphorylated Histon2A foci, nor did it change the hypersensitivity of *Ercc1* deficient cells to the DNA crosslinker MMC. It only affected the survival of cells, which is expected to have major consequences for leukemogenesis. Indeed, transplantation of BM lacking *Ercc1* and heterozygous for *Trp53* efficiently induced leukemia, whereas mice transplanted with $Trp53^{+/-}$ BM remained healthy, and $Ercc1^{-/d}$ BM was impaired in its engraftment capacity.

The observation that defective ICL repair underlies BM failure in *Ercc1* deficient mice render these mice a good model for Fanconi Anemia (FA). Several genes can be mutated in FA and their transcribed proteins constitute the FA-pathway which is involved in DNA repair at stalled replication forks. A particular problem in FA is the anemia due to unresponsiveness to erythropoietin (Epo). It is controversial whether this is due to the underlying DNA repair deficiency or an independent aspect of FA. We analyzed Epo signalling in erythroblasts derived from mice lacking *FancA*, *FancG* or *Ercc1* (**Chapter 5**). At atmospheric oxygen levels $Fanca^{-/-}$ and $Fancg^{-/-}$ erythroid progenitors are impaired in their expansion capacity and show impaired Epo-induced Stat5 and Erk1/2 phosphorylation. However SCF-induced signal transduction and Epo-induced phosphorylation of the Epo-receptor and Jak2 were not affected, suggesting that the signaling defect occurs distal of the Epo-receptor/Jak2 complex. We also saw this defective signal transduction in wild type erythroid progenitors that were challenged with a sub lethal dose of the DNA crosslinkers Mitomycin C (MMC) and in $Ercc1^{-/-}$ erythroid progenitors . Thus, the signalling defect is not specific for cells lacking specific Fanconi proteins. Interestingly impaired Epo-induced Stat5 and Erk phosphorylation upon MMC treatment of erythroblasts was p53 dependent. Therefore, attenuated Epo signaling is caused by

accumulating ICLs and may contribute to Epo-resistance in the anemic phase of FA. It suggests that an impaired response to cytokines, which is often seen in BM failure syndromes, may contribute to impaired progenitor proliferation, but it is a secondary event to the activation of p53 and seems to be part of a more general senescence program.

So far we identified p53 as a major player that restricts HSPC proliferation when DNA damage stalls replication (**Chapters 4 and 5)**. Since activated p53, or its related pathways, limit transient amplification, we wondered which factors abrogate this function to restore the proliferative capacity of cells and how such factors contribute to leukemogenesis. Wnt factors enhance transient amplification and may alter the function of tumor suppressor pathways (**Chapter 6**). Erythroblasts express low levels of various Wnt factors. In these erythroblast cultures p53 limits the transient expansion capacity. We showed that p53 controls β-catenin stabilization. Our data suggests that p53 activation and Wnt signalling may act as antagonizing pathways that control progenitor expansion.

Relevance of Ercc1 deficient mice as a model system for bone marrow failure and subsequent leukemogenesis

The aberrant function of transcription factors due to translocations of the encoding genes such as t(8:21) or t(15:17) usually lead to a block in differentiation and factor-independent proliferation of progenitors [8]. These types of leukemias respond well to genotoxic agents. In contrast, the response to cytotoxic therapy is much less in leukemias arising from congenital bone marrow failures caused by genotoxic lesions or genotoxic therapy related secondary leukemias. This can be explained by the fact that these particular leukemias arise from mutations in gatekeeper genes (tumor suppressors). Initially mutations in these genes cause stress induced premature senescence (SIPS), which has to be overcome by additional mutations before cancer can occur. These leukemias respond poorly to cytotoxic therapy, because therapy is based on activation the same tumor suppressor systems.

Several hereditary cancer syndromes, among which Fanconi Anemia (FA), Seckel syndrome and Bloom syndrome, initially develop bone marrow failure which progresses towards leukemia over time. All of the mentioned diseases are defective in the recognition as well as the processing of replication dependent lesions such as interstrand crosslinks (ICLs) which are induced by Mitomycin C (MMC) and Cisplatin [9]. This situation is not the only one in which an aplastic bone marrow progresses into leukemia.

Similar events occur in secondary or therapy-related MDS (t-MDS) and AML (t-AML). Drugs used to treat solid cancers (such as Cisplatin and Pol-II inhibitors) frequently cause t-MDS resulting in subsequent leukemia [10]. Tumor suppressor genes deregulated in these cases are usually *CHK1* [11], *CHK2* [12], *p53* [13] and *p16* [14].

Mice deficient in *FancC* are used to model this subset of leukemias. Hematopoietic progenitors as well as hematopoietic stem cells isolated from these mice have impaired functionality *in vitro* and show an increased sensitivity to MMC [15-16]. Interestingly this is only the case in progenitors isolated from adult BM and not from juvenile BM, indicating a progressive hematopoietic defect. This is confirmed by the fact that BM cells isolated from *FancC* deficient mice have significantly reduced multi-lineage repopulating ability in competitive transplantation assays (short as well as long term) [15, 17-18]. Transplanted mice display a MDS like phenotype. However the overall primary phenotype of *FancC* deficient mice is relatively mild and spontaneous leukemias are not observed. For studying the transition of BM failure to leukemia it would be of use to employ a model which does develop leukemias without using bone marrow transplantation settings.

To study the transition of BM failure to leukemia development we choose to utilize an *Ercc1* deficient mouse model. The Ercc1-Xpf heterodimer functions as an endonuclease in NER as well as ICL repair. Mice lacking this gene develop the characteristics of bone marrow failure (similar to FA) at a very young age (3 weeks postnatal) as well as signs of progeria [7]. However their overall phenotype is much more severe and therefore this model is more robust than the Fanconi mouse models.

The balance between aging and cancer is tightly associated with the type of DNA damage that is encountered as well as the level of damage. Although DNA damage can be expected to impair cell growth in general, different DNA repair deficiencies result in distinct phenotypes. As we showed in chapter 2 defective ICL repair in *Ercc1* deficient mice results mainly in a hematopoietic defect while the NER defect contributes to their aging phenotype. As is hypothesized in FA, the ICL load is of great importance for the development of an aplastic bone marrow. Since mice completely defective in *Ercc1* show such a severe phenotype that they do not survive weaning, this model is not suitable for studying leukemogenic development. However mice that are hypomorph for *Ercc1* do survive for longer and show the potential to become leukemogenic.

This effect in the dosage of *Ercc1* has been described in chapter 3 of this thesis. Although it is not unlikely that deficiencies in other repair genes can also lead to such differences in phenotype. This reduced activity of caretakers can result in a pre-leukemic environment in which additional hits can lead to the development of clonal cell populations which can uncontrollably proliferate.

Leukemia types derived from an aplastic bone marrow environment

The type of leukemia that mainly develops in FA patients is acute myeloid leukemia (AML), only very rarely acute lymphoid leukemia (ALL) develops [19]. This suggests that the main hematopoietic defect is localized in the myeloid compartment. Which is in line with our findings in chapters 2, 3 and 4 where we show that in FACS analysis of total BM the CMPs as well as the GMPs are affected (in numbers as well as functionality) by the loss of Ercc1 while the CLP compartment is not influenced.

In mouse models for leukemia development, mainly ALL develops. For example, in mice lacking p53 mostly lymphoma's are formed [20]. In our *Ercc1*$^{-/d}$*Trp53*$^{+/-}$ transplantation model the recipient mice developed heterogeneous leukemia's (Chapter 4). The mice suffered from increased spleen and liver but leukemic BM cells also carried lymphoid markers such as CD3e and CD19. Loss of 1 allele of *Trp53* increases the likelihood that these cells will completely lose *Trp53* expression due to a secondary mutation. However a complete loss of *Trp53* functionality itself is not sufficient for leukemic development as we showed that erythroblasts deficient in both *Ercc1* and *Trp53* are still capable of H2AX phosphorylation upon ICL induction and are also still completely growth factor dependent in addition to unchanged proliferative capacity (Chapter 4). Therefore loss of *Trp53* expression in an *Ercc1* deficient background will only facilitate mutagenesis by abrogating the senescence response to DNA damage but will not immediately contribute to factor independent proliferation or a block of differentiation. However it is still unclear which of the downstream targets of *Trp53* is critical for the regulation of the hematopoietic stem cell response to continuous ICL damage. Our model could prove useful to identify the different players and their regulatory pathways by using next generation sequencing approaches in the different stages of leukemic development. By identifying key players this information may be used to develop novel strategies that could be widely applicable to cancers arising from a genotoxic background.

Mechanism of Trp53 activation upon detection of ICLs

Bone marrow failure in FA patients has been specifically linked to the extreme sensitivity of bone marrow cells to oxidative damage [21]. Primary cells from FA patients are uniquely sensitive to oxidative stress induced DNA damage and the following p53 induced growth arrest [22]. Although p53 induced growth arrest prevents the outgrowth of cells that accumulated damaged DNA, it also leads to depletion of stem and progenitor cells and subsequent bone marrow failure. In a hypoxic environment *Fanc* deficient cells proliferate much better, which may be caused by a decrease in culture specific oxidative damage.

However, there is also a possibility that lower cell division rate plays a role. The specific signaling pathway activated upon ICL detection is *Trp53* dependent. Upon replication fork stalling, increasing amounts of single stranded DNA are formed that bind ATRIP, resulting in the activation of ATR kinase [23]. This will lead to phosphorylation of H2AX and subsequent activation of downstream effectors such as Chk1, Brca1 and p38. Phosphorylation of p53 by p38 or Chk1 will stabilize the protein, whereas Brca1 acts as a scaffold for the recruitment of DNA repair enzymes that can remove the crosslink by either homologous recombination or non-homologous end joining. The requirement for stalled replication forks was supported by experiments in which we exposed cells for increasing time intervals to MMC. Short exposure to MMC does not influence cell viability and signaling, however prolonged exposure (>16hr) or exposure to high dosages leads to increased phosphorylation of Chk1. The number of cells with phospho-Chk1 foci in the nucleus correlated to the number of cells expected to reach S-phase in the same time interval (J.Verhagen-Oldenampsen. unpublished).

Recently it has been shown that spontaneous abrogation of the G2 checkpoint in a subset of FA patients indeed promotes leukemogenesis [24]. These patients expressed only very low levels of Chk1 and p53 thereby preventing p53 induced cell death. Due to this they displayed only mild bone marrow deficiencies (near normal blood counts) and as a result reached adulthood. However, several patients developed myelodysplasia and/or leukemia thus it is likely that these abrogated cell subsets contribute to leukemogenesis by promoting cell survival in a genetic background of constitutive genetic instability.

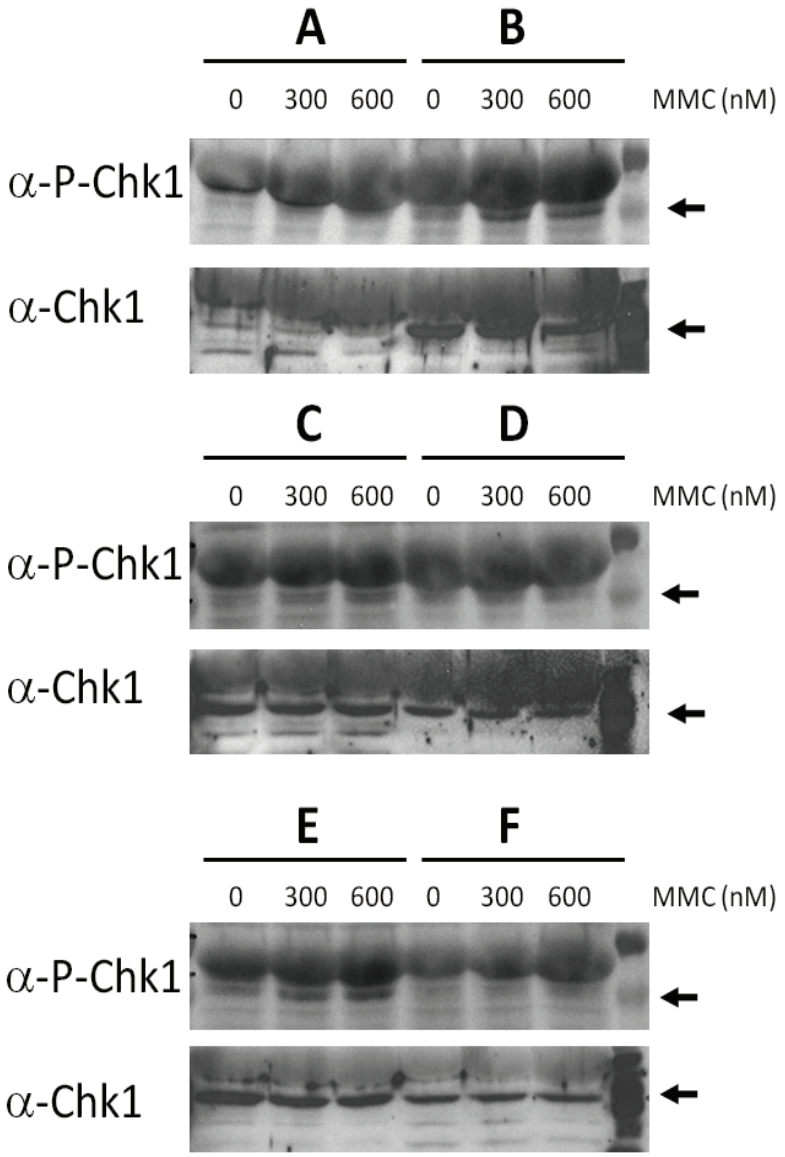

Figure 1: *CHK1 expression and phosphorylation is heterogeneous in primary human AMLs with a normal karyotype. Primary AML samples were defrosted and cultured under proliferative conditions. Cells were exposed overnight to MMC and harvested the next day. Cell lysates were made and blots were stained with anti-PChk1 and total Chk1.*

Role of other tumor suppressor genes in bone marrow failure and leukemogenesis

Chemotherapeutic drugs, such as MMC, activate multiple pathways in addition to p53-induced targets, such as p73 activation by CHK1/CHK2 and subsequent transactivation of PUMA [25]. Also MAPKs such as p38 and FOXO1 [26] couple DNA damage to caspase-3 activation. In p53 proficient cells MMC induces an increase in p53 protein which leads to activation of p53 regulated genes and apoptosis. Many cancer cells are p53 deficient and rely on the ATR/CHK1 checkpoint. If CHK1 would become inactive (e.g. due to mutations) these cells would become more sensitive to caspase-2 mediated cell death. In a recent study on DNA damage signaling in AML it was found that high levels of DNA damage signaling were present (phosphorylation of H2AX and CHK1) in complex karyotype AML samples and that this is already the case early on in leukemogenesis [27]. Upon Inhibition of CHK1 in these AML samples the authors found that progenitors with high DNA damage loads were sensitized to AraC treatment while normal progenitors were unaffected. The same protective effect of CHK1 against DSBs was seen in BCR/ABL leukemia cells which were treated with DNA crosslinking agents [28]. Therefore it seems that the checkpoint kinases pose an interesting target for cancer therapy.

In addition, CHK1 inactivation may also be a step in leukemogenesis. In preliminary experiments we examined MMC-induced CHK1 phosphorylation in AML samples with a normal karyotype (Figure 1). This showed that patients differed in their sensitivity for MMC induced CHK1 phosphorylation. In two out of six samples (B, E) MMC induced strong phosphorylation of CHK1. Two others showed weak phosphorylation of CHK1 (C, D), whereas in two others MMC failed to induce any pCHK1 (A, F). Analysis of total CHK1 levels revealed that CHK1 was not expressed in sample A, whereas in sample F levels were normal, indicating that the upstream phosphorylation mechanism was abrogated. This shows that there is a high degree of heterozygosity between patients in their DNA damage response, and thus individual treatment regimes are of high importance.

$p19^{Arf}$ ($p14^{ARF}$ in humans) and $p16^{INK4a}$ are both encoded by the *Ink4a/Cdkn2A* locus [29]. $p16^{INK4a}$ impacts on the Rb pathway [30] while $p19^{Arf}$ targets the p53 tumor suppressor pathway [31]. This interaction is indirectly due to its interaction with Mdm2. As we showed in chapter 4, loss of the *Cdkn2a* locus did not affect the phenotype of *Ercc1* deficient mice, since deletion of this gene did not improve their severe hematopoietic phenotype. Interestingly, this also implies that $p19^{Arf}$ is dispensable for p53 activity in the BM of $Ercc1^{-/d}$ mice.

Loss of both $p16^{INK4a}$ and $p19^{Arf}$ has little effect on HSC function since there is only a very subtle increase in serial repopulating ability [32]. In freshly isolated BM cells $p19^{Arf}$ is not expressed but it is induced during culture. The induction of $p19^{Arf}$ during culture impairs the formation of very early CFU-S colonies [32]. However in MEFs deletion of both proteins did effect proliferation capacity (Chapter 4), suggesting that the function of $p16^{INK4a}$/$p19^{Arf}$ is cell type and tissue specific.

p38 MAP kinases respond to a variety of cellular stress stimuli, including UV radiation and hypoxia [33]. They are also involved in developmental hematopoiesis. Mice lacking p38α are embryonic lethal because they have defects in placental angiogenesis and p38 inactivity is linked to a decrease in Epo gene expression [34-35]. In *FancC* deficient MEFs and progenitors, p38 inhibition has been shown to ameliorate apoptotic responses [36] and recently it was shown that p38 inhibitors improve multi lineage repopulating ability of *FancC* deficient HSCs [37]. There is a link between Cisplatin induced DNA damage (interstrand crosslinks) and p38 activation, NER defective mutants show higher levels of JNK and p38 activation compared to repair proficient cells [38]. Activation of p38 results in phosphorylation of human p53 on Ser33 and Ser46 after UV radiation [39], and on Ser15 and Ser392 following DSB [40]). This phosphorylation leads to dissociation of p53 from MDM2 resulting in accumulation of p53 protein. In primary human erythroid progenitors the p38α isoform is involved in induction of differentiation but not in apoptosis or cell proliferation [41]. We tested whether inhibition of p38 could enhance transient proliferation of *Ercc1*-deficient erythroblasts. The p38 inhibitor SB203580 indeed completely rescued the abrogated proliferation of *Ercc1*-deficient erythroblasts, however this was not specific for loss of *Ercc1*. In primary mouse erythroblasts we saw that inhibition of p38 by SB203580 alleviated the spontaneous differentiation of *wt* cells as well, and induced extended proliferation similar to loss of *Trp53*. Thus p38 and p53 seem to function in the same pathway in erythroid cultures (Preliminary data, Figure 2).

Role of Wnt signalling in hematopoiesis and leukemogenesis

Wnt signal transduction is very important for maintaining the balance between proliferation and differentiation throughout embryogenesis as well as postnatal life [42-43]. Its role in stem cell maintenance is especially well characterized and as such it can be regarded as antagonistic pathway of gatekeepers, since this pathway stimulates longevity of stem cells and increases the number of progenitor cells at the increased risk of tumor development. Enhanced Wnt signaling has been associated with may cancer types such as colon cancer [44], breast cancer [45], melanoma [46] as well as leukemia [47].

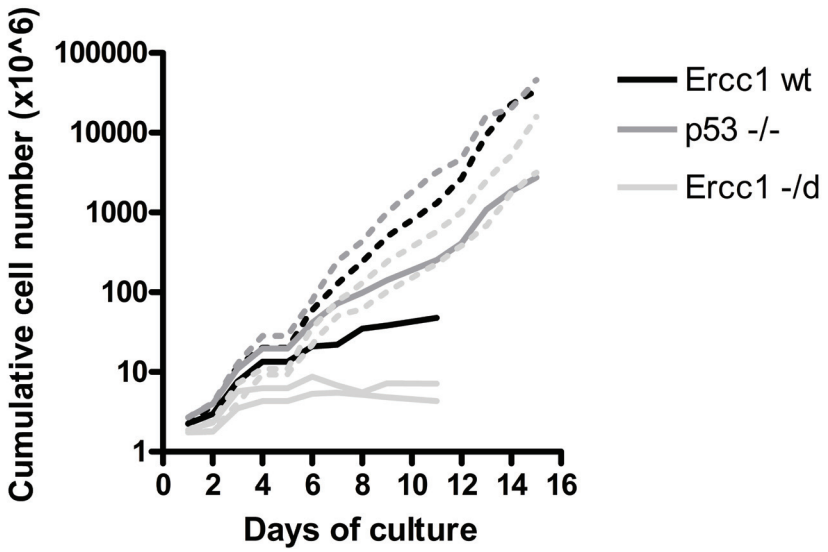

Figure 2: *p38 inhibitor prolongs proliferation of primary erythroblasts irrespective of their genotype. Primary erythroblasts were cultured under renewal conditions and counted daily. Dotted lines indicate addition of p38 inhibitor SB203580.*

Under genotoxic stress conditions p53 is activated and a pathway for β-catenin degradation is initiated. p53 can directly induce *Siah-1* expression and in turn formation of a unique SCF-like complex (SCF(TBL1)) consisting of Siah-1, Siah-1 interacting protein (SIP), SKP1, transducinβ-like1 (TBL1) and APC [48-49]. Siah-1 (as a critical protein in this pathway) can efficiently deplete β-catenin, thereby downregulating transcription of Wnt target genes. In addition, Siah-1 also functions as an E3 ligase which is able to directly bind and poly-ubiquitinate β-catenin *in vitro*, although this interaction is very weak [50]. p53 overexpression induces transcriptional activation of *Siah-1* genes in a variety of mammalian cell lines [51-53] and overexpression of Siah-1 can mimic the effects of p53 activation by inducing cell cycle arrest or apoptosis [54]. Following DNA damage, the p53 mediated induction of Siah-1 induces degradation of β-catenin independently of the GSK3β mediated pathway. Since overexpression of β-catenin promotes cell cycle progression and inhibits cell cycle arrest induced by gamma irradiation [55], Siah-1 mediated β-catenin degradation may contribute to p53 dependent cell cycle arrest. Interestingly, accumulation of a β-catenin

mutant protein that is resistant to degradation by the GSK3β pathway induces activation of p53 [56]. Since overexpression of Siah-1 can induce degradation of wildtype or mutant forms of β-catenin, Siah-1 may function downstream of p53 in a pathway that senses and degrades oncogenic β-catenin and thereby contributes to tumor suppression.

The Wnt/β-catenin pathway is active in certain types of leukemia [57-60] as well as in normal HSCs [61-62]. The group of Zhao *et al* has shown that different β-catenin levels lead to varying effects in a BCR-ABL model of leukemogenesis [63] . Using conditional β-catenin deficient mice they showed that loss of β-catenin expression from the hematopoietic system decreased the incidence of CML. Interestingly they could rescue this phenotype by ectopically expressing low levels of β-catenin whereas high levels of β-catenin had adverse effects on leukemogenesis. This shows that the level of β-catenin expression as well as the activation status is of utmost importance for either its pro- or anti tumorigenic function. To date no β-catenin mutations are reported in AML or CML however via other ways it is still possible that deregulation of the Wnt/β-catenin pathway is involved in leukemogenesis. For example, increased FLT3 signaling (due to mutations or amplification) can lead to AKT mediated phosphorylation (and thus inactivation) of GSK3β. This will lead to stabilization of β-catenin and thus to increased Wnt signaling [64]. Certain *Flt3* mutations lead to an increase in Frizzled-4 expression and also increase β-catenin signaling [65]. Translocation products such as AML-ETO and PLZF-RARα can activate the Wnt/ β-catenin pathway by activating plakoglobins [66]. And lastly it is reported that abnormal promoter hypermethylation of Wnt inhibitors (such as DKK3) can enhance Wnt/ β-catenin signaling in ALL and AML [67-68].

Many different types of animal models have been developed for the Wnt signaling pathway. However many conventional knockout mice were embryonic lethal and thus studying the exact role of the Wnt factors was only possible since the development of conditional mouse models (using cre-lox technology). Today, mouse models are available for almost all Wnt genes and many of them are well characterized (for a list of their phenotypes see the Wnt homepage [69]).

Erythroid progenitors isolated from *wt* mouse fetal livers can undergo a limited number of renewal divisions while *Trp53* deficient erythroblasts can undergo indefinite renewal divisions without increased differentiation. We observed that *Trp53* deficient erythroblasts display high levels of nuclear β-catenin while *wt* erythroblasts only express very low levels (Chapter 6). In *Ercc1* deficient cultures we also found very low to absent levels of β-catenin which is interesting

since these cells show increased spontaneous differentiation compared to *wt* cells. It would be interesting to see if it is possible to alleviate the amount of spontaneous differentiation in *Ercc1* deficient erythroblast by introducing Wnt factors into the culture. For this purpose we aimed to transduce *Ercc1* deficient erythroblasts with a bi-cistronic retroviral vector harboring either *Wnt3a* or *Wnt5a*. While this process was successful in *wt* erythroblast (Chapter 6) we were unable to transduce *Ercc1* deficient erythroblast using this method. Probably these cells are already so fragile by their inability to repair damage that the process of transduction inevitably induces apoptosis in these cells.

To circumvent this problem we decided to generate a conditional mouse model in which we are able to selectively choose when and where we activate the Wnt signaling pathway. Oocytes were injected with the pE/I vector carrying floxed *eGFP* in front of a *Wnt5a-HA* sequence, all under the control of the β-*actin* promoter. Without cre recombinase expression only eGFP will be expressed while in the presence of cre expression eGFP will be floxed out and WNT5a-HA will be expressed (see Figure 3 for a schematic representation and functionality tests). By using cre recombinase expressed under different promoters it is possible to regulate the place and timeframe of Wnt5a-HA expression. First we crossed the transgenic *Wnt5a-HA* mice with mice which expressed cre recombinase from the *Cag* locus [70]. This is a general germline promoter and Wnt5a-HA is expected to be expressed early on in all tissues. Using this technique 25% of the offspring is expected to carry the transgene, unfortunately only 2 viable pups harboring the vector were born out of 47 pups in total (4%). Analysis of these mice indicated that there was no expression of Wnt5a-HA. It seems very likely that the cre recombinase expressed from the *Cag* locus is to abundant which leads to embryonic lethality of the embryos carrying the transgene. As a second approach we crossed the transgenic *Wnt5a-HA* mice with a cre recombinase expressed specifically in the hematopoietic system (*C/EBPα*-cre). C/EBPα is expressed in a significant subset of multipotent hematopoietic progenitors which predominantly give rise to the myeloid lineage during steady state hematopoiesis [71]. In this setting the *C/EBPα* cre mice were crossed with *YFP* mice so that progeny of the cells which expressed cre is easy to trace using flow cytometry. Pups were born in the mendelian frequency, yet pups carrying the transgene showed no hematopoietic phenotype and no Wnt5a expression. Further work will be necessary to determine why no effect of Wnt5a expression is seen. Possibly the *Wnt5a-HA* transgene is non-functional or autocrine expression of Wnt5a has no effect on steady state hematopoiesis. Further research will be necessary to determine what the exact reason is, currently TOP/FOP reporter vectors are being used to transduce stroma cell lines on which *C/EBPα*-cre YFP^{+} *Wnt5a-HA* cells can be plated to determine the YFP expression in stroma cells.

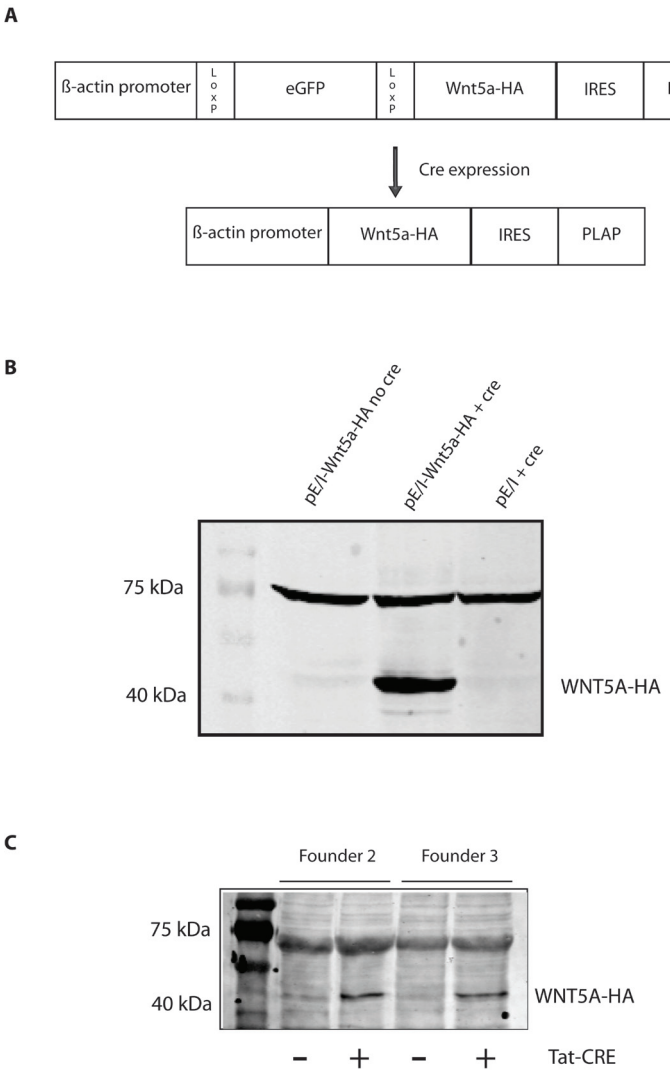

Figure 3: *Wnt5a-HA transgenic construct and testing in vitro and in vivo.* **(A)** *Schematic representation of the construct used for oocyte injections.* **(B)** *In vitro testing of the construct used for oocyte injection. Transient transfection of HELA cells with the pE/I-Wnt5a-HA construct with or without the addition of cre recombinase.* **(C)** *In vivo testing of construct activity. Bone marrow was harvested from two founder animals. Cells were cultured under erythroblast renewal conditions with or without the addition of Tat-Cre.*

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Nederlandse samenvatting
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NEDERLANDSE SAMENVATTING

Gedurende het hele leven van een organisme wordt het DNA blootgesteld aan condities die nucleotiden modificeren of de DNA strengen breken. Deze DNA schade veroorzaakt veroudering op het niveau van cellen en weefsels maar ook in het gehele organisme. Incorrect of foutief DNA herstel veroorzaakt mutaties welke kunnen leiden tot kanker. Het is daarom niet verbazend dat de incidentie van kanker toeneemt in oudere individuen.

Er is al veel bekend over syndromen die gerelateerd zijn aan gebrekkig DNA schade herstel, er is echter weinig bekend over waarom bepaalde specifieke DNA schade herstel gebreken leiden tot specifieke fenotypes. Het DNA schade herstel netwerk bestaat uit een complex netwerk van eiwitten welke verantwoordelijk zijn voor herkenning van schade, signaal transductie, regulatie van de celcyclus en DNA herstel. Dit complexe netwerk zorgt voor een strikte balans tussen veroudering van het organisme en het ontstaan van kanker.

De cellen in ons bloed hebben een relatief korte overlevingstijd, om het aantal bloedcellen constant te houden is er een doorlopende productie van nieuwe cellen nodig vanuit de hematopoietische stam- en voorlopercellen (HSPCs) in het beenmerg. Door deze hoge delingsgraad is het hematopoietisch systeem zeer gevoelig voor geneesmiddelen die het DNA beschadigen. Cytotoxische anti kanker geneesmiddelen die veelvuldig gebruikt worden voor diverse soorten kanker hebben meerdere schadelijke neveneffecten op de hematopoiese. Het langdurig behandelen met een hoge dosering alkylerende middelen (bijv. cyclofosfamide gebruikt bij o.a. borstkanker behandeling) kan leiden tot een toenemende depletie van HSPCs met beenmerg hypoplasie als gevolg. Door deze hypoplasie kunnen de overgebleven HSPCs aangezet worden tot deling terwijl de mutaties, ontstaan door DNA schade in deze cellen, kunnen leiden tot een verhoogd risico op het ontstaan van leukemie.

De experimenten beschreven in dit proefschrift hadden tot doel de moleculaire mechanismen van beenmergfalen en de hierop volgende leukemogenese, zoals klinisch waargenomen in syndromen met een falend beenmerg door veroudering of aanhoudende DNA schade te identificeren. Voor deze experimenten hebben we muizen gebruikt die deficiënt zijn voor *Ercc1*. *Ercc1* is een endonuclease betrokken bij de excisie van nucleotiden tijdens globaal en transcriptie gekoppeld nucleotide excisie herstel (NER) alsmede bij de excisie van crosslinks (ICL) tussen de twee DNA strengen

bij een replicatievork. Voorgaande studies hebben laten zien dat het aantal hematopoietische stamcellen in het beenmerg van Ercc1 deficiënte muizen verlaagd is, en dat de voorloper cellen verminderd in staat waren om kolonies te produceren.

In deze studie hebben we eerst bekeken of de ernstige veroudering (progeria) van de gehele muis, welke geassocieerd is met een verminderde NER functie, bijdraagt aan hun beenmergfalen of dat dit specifiek gerelateerd is aan een verminderde ICL reparatie op de replicatievork (**Hoofdstuk 2**). Uit een vergelijking van *Ercc1*$^{-/-}$ muizen met *Xpa*$^{-/-}$*Csb*$^{-/-}$ muizen, welke een geheel gebrek aan NER hebben maar wel functioneel zijn voor ICL reparatie, bleek dat de ernstige progeria in *Ercc1*$^{-/-}$ muizen niet bijdraagt aan het verouderen van het beenmerg compartiment. De alternatieve verklaring, dat HSPCs vooral gevoelig zijn voor defecten in ICL reparatie werd ondersteund door het feit dat de “quiescent” stamcellen van de HSPC populatie relatief gezien minder aangedaan waren zodra de muizen volwassen waren. Zowel conditionele knock out muizen als hematopoietische repopulatie studies waarin *Ercc1* deficiënt beenmerg werd getransplanteerd in bestraalde ontvangers wezen uit dat er een progressief verlies is van *Ercc1* deficiënte cellen. Dit is in overeenstemming met de hypothese dat proliferatie nadelig is voor cellen met een verminderde DNA reparatie capaciteit.

Lange termijn hematopoiese kon niet onderzocht worden in *Ercc1* muizen doordat ze drie weken na de geboorte overlijden. Muizen met één “hypomorph” allel (*Ercc1*$^{-/d}$) leven ongeveer 20 weken en muizen met twee “hypomorph” allelen (*Ercc1*$^{d/d}$) leven gemiddeld langer dan 6 maanden. Dit verschil in overlevingstijd moet bijna wel komen door een doserings effect van hun resterende *Ercc1* activiteit. Daarom hebben we vervolgens onderzocht hoe kleine verschillen in *Ercc1* activiteit parameters van progeria en hematopoiese beïnvloedden (**Hoofdstuk 3**). Alhoewel zowel *Ercc1*$^{-/d}$ als *Ercc1*$^{d/d}$ muizen beide significant langer leven dan volledige *Ercc1* knockout muizen zijn ze nog steeds 50% kleiner dan hun wildtype nestgenoten. Ze vertonen ook tekenen van polyploïdie in de lever wat aangeeft dat ze vroegtijdig verouderen. Echter de mate van polyploïdie nam af als de mate van *Ercc1* activiteit toenam. LSK aantallen namen toe in het totale beenmerg van jonge dieren (3 en 10 weken oud) terwijl er geen verschil was in meer volwassen dieren van 20 weken oud. Hoogstwaarschijnlijk komt dit doordat er op 20 weken minder proliferatie plaatsvindt en derhalve er dus minder DNA schade opgelopen wordt welke gerepareerd moet worden. Binnen het LSK compartiment was er een verschuiving in het voordeel van de meer primitieve cellen (LT-HSC & ST-HSC) ten koste van

een lager aantal multipotente voorloper cellen. Deze verschuiving was zichtbaar in alle *Ercc1* deficiënte dieren en wordt geassocieerd met de voornamelijk "quiescent" status van primitieve stamcellen en de actieve delingsstatus van multipotente voorlopercellen. Binnen het voorlopercel compartiment is er een drastische afname van myeloïde voorlopers terwijl er geen effect is op de lymfoïde voorlopers. Alhoewel een laag niveau van Ercc1 het aantal voorlopers verhoogde waren deze cellen nog steeds niet in staat om BFU-E of CFU-G te vormen. Alleen het aantal CFU-GM was licht toegenomen in *Ercc1*$^{d/d}$ muizen maar wildtype aantallen werden nooit bereikt. Deze resultaten wijzen erop dat het niveau van *Ercc1* voornamelijk het verouderings fenotype van de *Ercc1* deficiënte muizen verbeterd terwijl het hematopoietische fenotype niet veranderd is.

Progeria veroorzaakt door onderdrukking van de hormonale groei-as is gekoppeld aan aanhoudende DNA schade en niet werkende TC-NER. Omdat we vastgesteld hadden dat het hematopoietisch defect onafhankelijk was van progeria en NER (**Hoofdstuk 2**) zijn we vervolgens gaan bekijken welke tumor suppressor genen verantwoordelijk zijn voor beenmerg veroudering (**Hoofdstuk 4**). De expressie van de p53 target genen *Puma* en *Noxa*, en de genen liggend op het *Cdkn2a* locus, p16^{Ink4A} en p19^{Arf} was verhoogd in *Ercc1*-deficiënte beenmergcellen ten opzichte van wildtype cellen. Deletie van de *Trp53* of de *Cdkn2a* locus in *Ercc1* deficiënte muizen bracht aan het licht dat p53 maar niet p16 of p19 bijdroeg aan de afname van het HSPC compartiment door gebrekkige ICL reparatie. Fascinerend genoeg bleek dat verlies van p53 de proliferatie capaciteit van hematopoietische voorlopers verhoogde, maar dat het de segmentale progeria en lever polyploïdie in *Ercc1* deficiënte muizen werd verergerd. De primaire moleculaire response op vastgelopen replicatie vorken, gemarkeerd door de formatie van gefosforyleerde histon2A foci, en de extreme gevoeligheid van *Ercc1* deficiënte cellen voor de DNA crosslinker MMC werden niet beïnvloed door het verlies van p53. Alleen de overleving van cellen werd beïnvloed, wat naar alle waarschijnlijkheid grote consequenties heeft voor leukemogenese. En inderdaad, transplantatie van *Ercc1* deficiënt, p53 heterozygoot beenmerg zorgde efficiënt voor leukemie ontwikkeling terwijl de muizen die getransplanteerd werden met *Trp53*$^{+/-}$ beenmerg volledig gezond bleven en *Ercc1*$^{-/d}$ beenmerg een verminderde repopulatie capaciteit liet zien.

De observatie dat gebrekkige ICL reparatie de onderliggende oorzaak is van beenmergfalen in *Ercc1* deficiënte muizen maken deze muizen zeer geschikt als model voor Fanconi Anemie (FA). Meerdere genen kunnen gemuteerd zijn in FA en hun bijbehorende eiwitten vormen de

FA-signaaltransductie route welke betrokken is bij DNA reparatie op vastgelopen replicatie vorken. Een veel voorkomend probleem in FA is anemie veroorzaakt door ongevoeligheid voor erythropoïetine (Epo). We hebben Epo signalering onderzocht in erythroblasten geïsoleerd uit muizen die deficiënt zijn voor *FancA*, *FancG* of *Ercc1* (**Hoofdstuk 5**). Bij atmosferische zuurstof niveaus zijn *Fanca*$^{-/-}$ en *Fancg*$^{-/-}$ erythroïde voorloper cellen verminderd in staat om te prolifereren en hebben ze een verminderde Epo-geïnduceerde Stat5 en Erk1/2 fosforylatie. De SCF geïnduceerde signaal transductie en Epo geïnduceerde fosforylatie van de Epo-receptor en Jak2 waren niet beïnvloed wat suggereert dat het signalerings defect zich distaal van het Epo-receptor/Jak2 complex bevindt. Hetzelfde defect werd gezien in wildtype erythroïde voorlopercellen die blootgesteld waren aan een subletale dosis van de DNA crosslinkers Mitomycin C (MMC) of Cyto-arabinoside (AraC). In *Ercc1*$^{-/-}$ erythroïde voorlopercellen, die in dezelfde DNA reparatie route werken, zagen we hetzelfde effect. Wat interessant is, is dat we ontdekten dat dit signalerings defect p53 afhankelijk was. Hierdoor is het mogelijk zo dat aanhoudende Epo signalering veroorzaakt door ophopende ICLs kan bijdragen aan Epo resistentie in de anemische fase van FA. Dus een verminderde respons op cytokines, wat vaak gezien wordt in syndromen met beenmergfalen, kan mogelijk bijdragen aan verminderde voorlopercel proliferatie, maar is een secundaire gebeurtenis na de activatie van p53 dat deel lijkt te zijn van een meer algemeen “senescence” programma.

Tot nu toe hebben we p53 geïdentificeerd als een belangrijke speler welke HSPC proliferatie beperkt wanneer DNA schade de replicatie stillegt (**Hoofdstuk 5**). Omdat geactiveerd p53, of de gerelateerde signalerings routes, kortdurende deling begrenst vroegen we ons af welke factoren deze functie van p53 kunnen opheffen om zo de proliferatie capaciteit van cellen te herstellen en hoe dit soort factoren kunnen bijdragen aan het ontstaan van leukemie. Wnt factoren versterken kortdurende deling en kunnen mogelijk de functie van tumor suppressor routes beïnvloeden en veranderen (**Hoofdstuk 6**). Erythroblasten hebben zeer lage expressie niveaus van Wnt factoren. In erythroblast kweken begrenst p53 de kortdurende expansie capaciteit. We laten zien dat p53 de stabilisatie van β-catenin reguleert. Onze data suggereert dat p53 activatie en Wnt signalering antagonistisch kunnen werken om voorlopercel expansie te reguleren.

ABBREVIATIONS

AA	Aplastic Anemia
ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
APC	Adenomatous Polyposis Coli
A-T	Ataxia Telangiectasia
ATF2	Activating Transcription Factor 2
A-T-LD	Ataxia Telangiectasia Like Disorder
ATM	Ataxia Telangiectasia Mutated
ATR	Ataxia Telangiectasia and Rad3 Related
ATRIP	ATR Interacting Protein
B-ALL	B-lineage Acute Lymphoblastic Leukemia
BER	Base Exchange Repair
BFU-E	Erythroid Burst-Forming Units
BioEpo	Biotinylated Erythropoetin
BM	Bone Marrow
Brca1	Breast Cancer 1
Brca2	Breast Cancer 2
BS	Bloom Syndrome
BSA	Bovine Serum Albumin
C/EPB	CCAAT/Enhancer Binding Protein
CDK4	Cyclin D1-Cyclin-Dependent Kinase 4
Cdkn2a	Cyclin-Dependent Kinase Inhibitor 2A
cDNA	Complementary DNA
CFU-G	Granulocytic Colony Forming Unit
CFU-GM	Granulocyte-Macrophage Colony Forming Unit
Chk1	Checkpoint Kinase 1
Chk2	Checkpoint Kinase 2
CLL	Chronic Lymphatic Leukemia
CLP	Common Lymphoid Progenitor
CML	Chronic Myeloid Leukemia
CMP	Common Myeloid Progenitor
CPD	Cyclobutane Pyrimidine Dimers
CS	Cockayne Syndrome
CSF3R	Colony Stimulating Factor 3 Receptor
DAPI	4’,6-Diamidino-2-Phenylindole
DBA	Diamond-Blackfan Anemia
DC	Dyskeratosis Congenita
DDR	DNA Damage Response

Dex	Dexamethasone
DNA	Deoxyribonucleic Acid
DSB	Double Strand Break
dsDNA	double strand DNA
Dsh	Dishevelled
DTT	Dithiothreitol
EMSA	Electrophoretic Mobility Shift Assays
Epo	Erythropoetin
EpoR	Erythropoetin receptor
Ercc1	Excision Repair Cross Complementing-group 1
Erk1/2	Extracellular Signal-Regulated Kinase 1/2
ESEP	Embryonic Stem Cell Derived Erythroid Progenitor
FA	Fanconi Anemia
FCS	Fetal Calf Serum
FL	Fetal Livers
Fz	Frizzled
G-CSF	Granulocyte Colony-Stimulating Factor
GG-NER	Global Genome NER
GH	Growth Hormone
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Monocyte Progenitors
GTP	Guanosine Triphosphate
H2AX	Histon 2AX
HR	Homologous Recombination
HSC	Hematopoietic Stem Cell
HSPC	Hematopoietic Stem- and Progenitor Cell
ICL	Interstrand Crosslinks
IGF-1	Insuline-Like Growth Factor
IgG	Immunoglobuline G
IP	Immune-Precipitated
IR	Ionizing Radiation
Jak2	Janus Kinase 2
Km	Kremen
LEF	Lymphocyte Enhancer Factor
LMPP	Lymphoid Primed Multi-Potent Progenitor
LRP	Low Density Lipoprotein Receptor-related Protein
LSC	Leukemic Stem Cells
LSK	(Lin-)Sca1+cKit+
LT-HSC	Long Term Repopulating Stem Cell
MDM2	Mouse Double Minutes 2

MDS	Myelodysplastic Syndrome
MEF	Mouse Embryo Fibroblasts
MEP	Megakaryocyte–Erythroid Progenitor
miRNA	microRNA
MLL	Mixed Lineage Leukemia
MMC	Mitomycin C
MMR	Mismatch Repair
MPP	Multi-Potent Progenitor Cell
mRNA	messenger RNA
NBS	Nijmegen Breakage Syndrome
NER	Nucleotide Excision Repair
NHEJ	Non-Homologous End-Joining
PBS	Phosphate Buffered Saline
PCNA	Proliferating Cell Nuclear Antigen
PCP	Planar Cell Polarity pathway
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
Q-PCR	Quantitative Polymerase Chain Reaction
Rb	Retinoblastoma
RBC	Red Blood Cell
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive Oxygen Species
RPA	Replication Protein A
RT-PCR	Real Time PCR
SCF	Stem Cell Factor
SCN	Severe Congenital Neutropenia
SDS	Shwachmann-Diamond Syndrome
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of Mean
shRNA	short hairpin RNA
Siah-1	Seven-In-Absentia homologue-1
SIP	Siah-1-Interacting Protein
SIPS	Stress Induced Premature Senescence
siRNA	small interfering RNA
SSB	Single Strand Breaks
ssDNA	single stranded DNA
Stat5	Signal transducer and activator of transcription 5
ST-HSC	Short Term Repopulating Stem Cell

T-ALL	T-lineage Acute Lymphoblastic Leukemia
tAML	therapy related AML
TCF	T-Cell Factor
TC-NER	Transcription Coupled NER
tMDS	therapy related Myelodysplastic Syndrome
TSG	Tumor Suppressor Genes
TTD	Trichothiodystrophy
UV	Ultraviolet
WBC	White Blood Cell
WCL	Whole Cell Lysates
WT	Wildtype
XP	Xeroderma Pigmentosum
γH2AX	phosphorylated Histon 2AX

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JO

CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 1 juni 1981 te ’s-Gravenhage. Zij behaalde haar atheneum diploma in 1999 aan het Atlas College, te Rijswijk. Vervolgens heeft zij haar doctoraal Bio-Farmaceutische Wetenschappen in 2005 behaald aan de Universiteit Leiden. Tijdens deze opleiding heeft zij 1 jaar *in vivo* onderzoek gedaan naar de dynamiek van cel adhesie eiwitten tijdens renale schade en regeneratie onder leiding van Prof. Dr. B. Van de Water, Dr. M. De Grauw en Dr. M. Alderliesten. Aansluitend hierop heeft zij nog 11 maanden onderzoek gedaan binnen TNO Quality of Life te Leiden, naar de functie van C-type lectinen in een *in vivo* model voor auto-immuun encephalomyelitis (EAE) onder begeleiding van Dr. L. Nagelkerken en Dr. J.M. Kel. In augustus 2005 is zij begonnen als promovenda in de groep van Dr. M. Von Lindern en Prof. Dr. I.P. Touw op de afdeling hematologie van het Erasmus MC. Het werk dat voor dit promotietraject is uitgevoerd, staat beschreven in het proefschrift dat nu voor u ligt. In april 2010 is zij begonnen als monitor bij het Clinical Research Bureau van de afdeling Maag-, Darm- en Leverziekten binnen het Erasmus MC. Sinds april 2011 is zij werkzaam als projectmanager binnen hetzelfde onderzoeksbureau.

PUBLICATIONS

Loss of Ercc1 results in a time- and dose-dependent reduction of proliferating early hematopoietic progenitors. **Judith H.E. Verhagen-Oldenampsen**, Jurgen R. Haanstra, Paulina M.H. van Strien, Marijke Valkhof, Ivo P. Touw and Marieke von Lindern. *Anemia 2012 Article ID 783068*

Critical role of p53 in interstrand-crosslink induced bone marrow failure and associated leukemic transformation . Jurgen R. Haanstra, **Judith Verhagen-Oldenampsen**, Paulina M.H. van Strien, Marijke Valkhof, Björn Schumacher, Ivo P. Touw and Marieke von Lindern. *Submitted*

Attenuated expansion and erythropoietin-induced signal transduction in *Fanca* and *Fancg* knockout erythroblasts is a p53-mediated response to defective repair of interstrand crosslinked DNA. Emile van den Akker, Henri J. van de Vrugt, **Judith Verhagen-Oldenampsen**, Godfrey Grech, Ivo Touw, Fré Arwert, Marieke von Lindern. *Submitted*

Wnt-signaling in erythroblasts is controlled by p53. **Judith Verhagen-Oldenampsen**, Sarah Duit, Pavel Vaclavik, Nuno Andrade, Ernst W. Müllner, Johannes Nimpf and Marieke von Lindern. *Submitted*

Extracellular signal-regulated kinase activation during renal ischemia/reperfusion mediates focal adhesion dissolution and renal injury. Maaike Alderliesten, Marjo de Graauw, **Judith Oldenampsen**, Yu Qin, Chantal Pont, Liesbeth van Buren and Bob van de Water . *Am J Pathol. 2007 Aug;171(2):452-62.*

Soluble mannosylated myelin peptide inhibits the encephalitogenicity of autoreactive T cells during experimental autoimmune encephalomyelitis. Junda Kel, **Judith Oldenampsen**, Mariken Luca, Jan Wouter Drijfhout, Frits Koning, Lex Nagelkerken. *Am J Pathol. 2007 Jan;170(1):272-80.*

PhD Portfolio Summary

Summary of PhD training and teaching activities

Name PhD student: Judith Verhagen-Oldenampsen	PhD period: 01-08-2005 to 1-2-2010
Erasmus MC Department: Hematology	Promotor: Prof. Dr. I.P. Touw
Research School: Molecular Medicine (MM)	Co-promotor: Dr. M. Von Lindern

1. PhD training		
	Year	**(ECTS)**
Courses		
- In Vivo Imaging – from cell to Organism	2005	1.8
- Basic and Translational Oncology	2005	1.8
- Annual Course Molecular Medicine	2006	0.7
- Biomedical Research Techniques V	2006	1.5
- Signal Transduction and Disease (MGC)	2006	1.1
- Genome Maintenance and Cancer (MGC)	2007	1.0
- Transgenesis and Gene Targeting (MGC)	2007	1.5
- Development, stem cells and disease (MGC)	2007	1.0
Workshops and Seminars		
- Workshop Molecular Therapeutics in Acute Leukemia	2005	0.5
- Workshop Browsing Genes and Genomes with Ensembl	2007	0.6
- Workshop Indesign	2011	0.3
- Erasmus Hematology Lectures	2005-2010	3.0
Oral Presentations		
- Hematology presentations	2005-2010	5.0
- Journal Club presentations	2005-2010	1.5
- 3rd Dutch hematology Congres	2009	0.5
- 4th Dutch hematology Congres	2010	0.5
Poster Presentations (5x)	2005-2010	3.0
(Inter) national conferences		
- 6 Symposia and Congresses	2005-2010	7.0
2. Teaching activities		
	Year	**ECTS**
Lecturing		
- Hematopoiesis course for 1st year Medicine Students	2007	0.5
- Supervising Master student Rotation (1 week)	2010	0.5
Total		33.3