Flt3 receptor tyrosine kinase in AML and its modulation of the p53/Hdm2/Bcl-2 pathway

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Scientific environment

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Abbreviations

AML	Acute Myeloid Leukemia
AML1-ETO	Acute myeloid leukemia-1 / eight-twenty-one
AraC	Cytarabine, cytosine β-D-arabinofuranoside
ATRA	All-trans retinoic acid
Bax	Bcl-2 Associated X protein
Bcl-2	B-cell lymphoma gene 2
DNR	Daunorubicin, daunomycin
Erk	Extracellular signal regulated kinase
FL	Flt3 ligand
Flt3	FMS-like tyrosine kinase 3 / Fetal liver tyrosine kinase 2 / FMS-like tyrosine kinase 3
FMS	Formerly McDonough feline sarcoma viral oncogene homolog
FRET	Fluorescense resonance energy transfer
GHR	Growth hormone receptor
GM-CSF	Granulocyte macrophage – colony stimulating factorLength mutation
Hdm2	Human homologue of murine double minute 2
HIF	Hypoxia-induible factor
HSCT	Hematopoietic stem cell transplantation
IDA	Idarubicin, idamycin
IR	Ionizing radiation
ITD	Internal tandem duplication
JM	Juxtamembrane
MAPK	Mitogen activated protein kinase
Mcl-1	Myeloid cell leukemia-1
PDGFR	Platelet-derived growth factor receptor
PI3K	Phosphatidylinositol 3-kinase
PML-RING	Promyelocytic leukemia / Retinoic acid receptor Really interesting new gene; distinct zinc-chelating domain involved in mediating protein-DNA and protein-protein interactions.
RTK	Receptor tyrosine kinase
SCF	Stem cell factor, Kit ligand, Mast cell growth factor, Steel factor
STAT	Signal transducer and activator of transcription
VLA	Very late antigen
WHO	World Health Organization
Y2H	Yeast-two-hybrid system

Summary

Acute myeloid leukemia (AML) is an aggressive disease with a poor overall survival rate. The most frequent mutations associated with AML are internal tandem duplications (ITD) in the juxtamembrane region of the receptor tyrosine kinase Flt3. These mutations render the receptor constitutively active and alter signaling trough *FLT3-ITD* compared to wild type receptor. Non-mutated *TP53* and elevated expression of its regulator Hdm2 is another interesting feature of AML. Studies have shown that the p53 pathway can be linked to Flt3 signaling as a subset of *FLT3-ITD* patients expresses increased levels of the anti-apoptotic protein Bcl-2 and hyper-phosphorylated p53 protein. This thesis investigates the properties of *FLT3-ITD* and the interconnection of the Flt3 signaling pathway with the p53/Hdm2/Bcl-2 pathway in an attempt to elucidate novel therapeutic targets in AML.

The experiments demonstrate a reciprocal regulation of Flt3 and Hdm2 in AML cells with Flt3-wt while *FLT3-ITD* cells suspend the Hdm2 modulation. *FLT3-ITD* patient cells have a higher level of Flt3 protein and the ITD results in dysregulated receptor turnover and attenuated Hdm2 down-regulation. Thus targeting of *FLT3-ITD* may result in elevated Hdm2 and increased tolerance for p53. The involvement of Bcl-2 family of proteins is also seen in this setting; a persistent attenuation of Mcl-1 is required for the cells to undergo apoptosis, however this is only seen in Flt3-wt cells. Hdm2 is in addition shown to be important as a regulator of Bcl-2. The blockage of Hdm2 E3 ligase activity results in increased Bcl2 and Hdm2 is required for Bcl-2 protection from p53-induced cell death.

To conclude, wild type and mutated Flt3 is interconnected with the important p53/Hdm2/Bcl-2 pathway at several levels. Therapeutic targeting of Flt3 should therefore be evaluated in co-operation with the p53/Hdm2/Bcl-2 pathway. Combined targeting of *FLT3-ITD*, Hdm2 and Bcl-2 may therefore be interesting when approaching the development of novel AML therapy.

List of papers

Paper I:

Bruserud Ø, Hovland R, Wergeland L, Huang TS, Gjertsen BT: **Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of Flt3-ligand effects in AML cell populations with and without genetic Flt3 abnormalities.** *Haematologica* 2003, **88:**416-428.

Paper II:

Wergeland L, Sjøholt G, Haaland I, Hovland R, Bruserud Ø, Gjertsen BT: **Pre-apoptotic response to therapeutic DNA damage involves protein modulation of Mcl-1, Hdm2 and Flt3 in acute myeloid leukemia cells.** *Mol Cancer* 2007, **6:**33.

Paper III:

Wergeland L, Oveland E, Sjøholt G, Bedringaas SL, Hovland R, Bruserud Ø and Gjertsen BT: **Flt3 mutations proximate to its ubiquitin dependent endocytosis motif suspend Hdm2 modulation.** *Manuscript.*

Paper IV:

Gjertsen BT, Wergeland L, Spurgers KB, Oveland E, Høiby T^2, Cascallo M, Honda T, Navone NM, Logothetis C, Lorens JB, Lozano G and McDonnell TJ: **Bcl-2 protects against p53-induced apoptosis through enhanced Hdm2 protein stability.** *Manuscript submitted.*

1. Introduction

1.1 Acute Myeloid Leukemia

Definition and classification

Acute Myeloid Leukemia (AML) is an aggressive hematological disorder in which the hematopoietic progenitor cells lose their ability to differentiate and proliferate normally (1). This leads to an accumulation of immature myeloid cells in the bone marrow (**Figure 1**).

Common symptoms of untreated AML are fatigue, bleeding due to thrombocytopenia, organ infiltration and fatal infections due to neutropenia, all resulting from the suppression of normal bone marrow function. The diagnosis of AML is based on the demonstration of an accumulation of myeloid blasts in the bone marrow. According to the French-American-British (FAB) cooperative group the diagnosis of AML requires at least 30% myeloid blasts in the bone marrow (2, 3). The more recent World Health Organization (WHO) classification defines AML as at least 20% leukemic blasts in the bone marrow. This last classification is based on morphology, histochemistry and cytogenetics. The WHO system defines four major categories of AML, namely; i) AML with recurrent genetic abnormalities, ii) AML with multilineage dysplasia, iii) therapy related AML and iv) AML not otherwise categorized (4, 5).

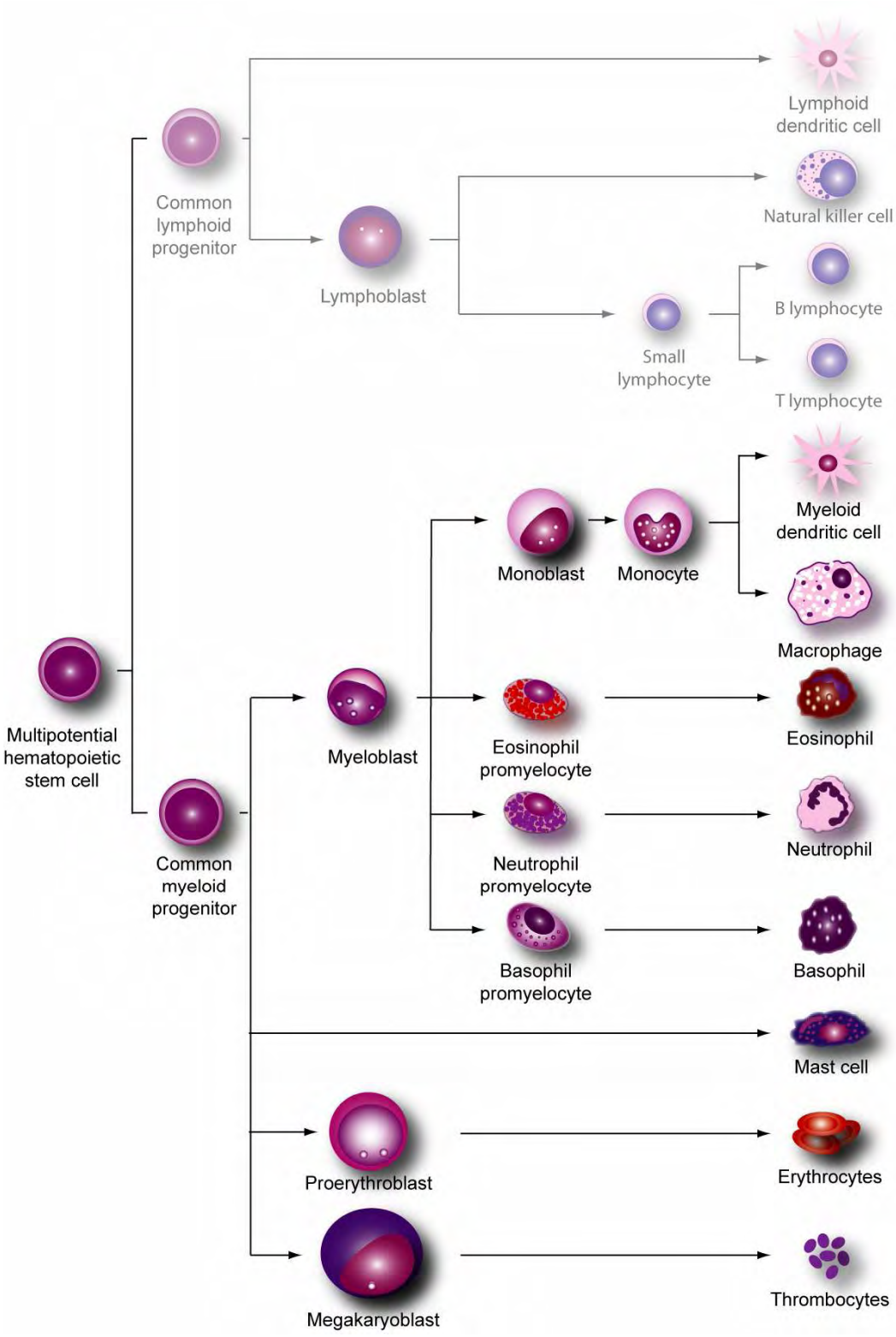


Figure 1. Normal hematopoiesis. *In normal hematopoiesis the multipotent hematopoietic progenitor cells differentiate into common lymphoid progenitors which further differentiate into the different lymphoid cells and the common myeloid progenitors which give rise to the myeloid cells. A block in maturation, increased proliferation and decreased cell death in the myeloid precursor cells lead to the development of AML.*

The current hypothesis of leukemogenesis is the "two-hit" model first presented by Gilliland in 2001 (6). This hypothesis implies that two separate mutations with different consequences need to be present for AML to develop. The first group of mutations is mutations giving a proliferative and /or survival advantage to the cell; referred to as class I mutations. Class I mutations include BCR-ABL, N-RAS, K-RAS, c-Kit (exon 8), c-Kit (Asp816), FLT3-ITD, FLT3 (Asp835), PTPN11, NF1 and TEL-PDGFRβ (7). The second group of mutations, the class II mutations, is mutations which impair differentiation and apoptosis; including CBFβ–MYH11, AML1–ETO, TEL–AML1, PML–RARα, NUP98–HOXA9, PU.1, C/CEBPα, AML1 and AML1–AMP19 (7). The "two-hit" model is overviewed in **Figure 2**.

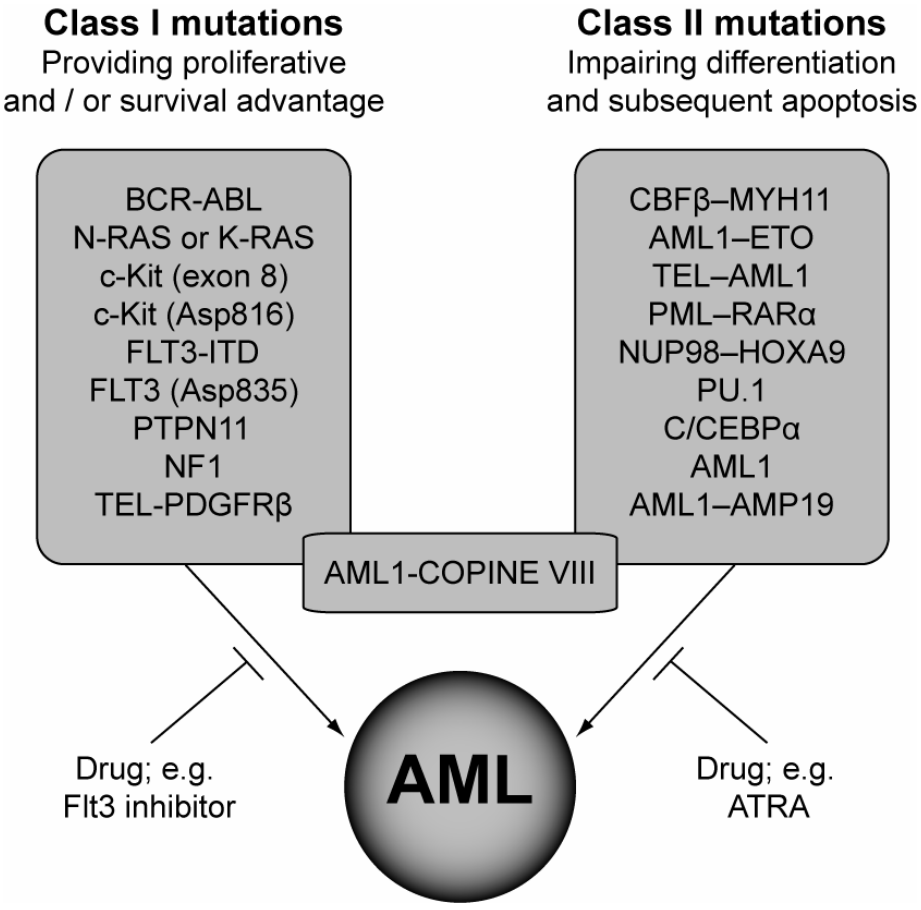


Figure 2 The "two-hit" model of AML development. *Adapted from (7, 8). Class I mutations include mutations which confer a growth advantage whereas class II mutations impair the hematopoietic differentiation. AML1-COPINE VIII is a unique mutation with a resulting fusion protein with both class I and class II activities (9).*

Prognostic factors

The median survival for AML patients receiving supportive therapy alone is only 3-4 months and very few patients survive for more than one year. There are numerous chromosomal aberrations and other genetic defects detected in AML, and recurrent cytogenetics abnormalities are used in prognosis and guidance for therapeutic decisions on hematopoietic stem cell transplantation or intensive chemotherapy alone (10-13). Based on the cytogenetics patients can be classified into three major subgroups with different prognosis. The group with favorable cytogenetics includes about 25% of the patients. Examples of favorable cytogenetics are t(8;21)(q22;q22) which creates the fusion protein AML1-ETO and t(15;17)(q22;q21) resulting in the PML-RARα fusion protein. Patients with this latter fusion protein receive targeted therapy with all-trans retinoic acid (ATRA) in addition to conventional therapy. The adverse cytogenetic group includes about 10% of AML patients with e.g. multiple abnormalities as deletions of either chromosome 5 or 7. There is also a group with intermediate risk; this includes among others the patients with normal karyotype. A detailed review of the cytogenetics in AML can be found in (12).

Several molecular genetic aberrations also have a prognostic impact in AML. The most important genetic aberration is in-frame internal tandem duplications (ITDs) of the receptor tyrosine kinase *FLT3* (14). Several point mutations in *FLT3* have also been described (15-20). *FLT3* mutations are associated with an adverse prognosis (1), and is the strongest separate marker for disease relapse in AML (21). A detailed description of *FLT3* mutations is given in a following section. Another frequent genetic aberration in AML is mutations in the nucleophosmin gene (*NPM1*). These mutations are present in 40 – 50% of AML patients with normal karyotype and represent a favorable prognostic factor for patients without *FLT3* mutations (22-26). Several other molecular prognostic markers have also been indicated, e.g.:

- Partial tandem duplications of the mixed lineage leukemia (*MLL*) gene associated with a short remission duration (27, 28).

- High expression of the Brain And Acute Leukemia Cytoplasmic (*BAALC*) gene is an adverse prognostic factor (29) .

- Mutations in CCAAT/enhancer binding protein alpha (*CEBPA*) gives a favorable prognosis of disease outcome (30).

- Over-expression of ETS-related gene (*ERG*), meningioma 1 (*MN1*) or breast cancer resistance protein (BCRP) has been shown to be predictors of poor prognosis (31-34).

- Mutations in *TP53* are associated with secondary leukemia and chemoresistance (35, 36)

A more detailed review of these molecular prognostic markers is given in (37, 38).

Gene expression profiling by DNA microarrays is a new method that is increasingly used for prognostic evaluation and identification of novel subclasses of AML (39). Gene expression profiling signatures have been correlated to clinical outcome in several studies (39-41) and will probably become a valuable tool for future molecular diagnostics.

Treatment

The initial treatment regimen for AML is induction therapy often consisting of an anthracycline (daunorubicine (DNR) or idarubicine (IDA)) in combination with cytosine arabinoside (AraC). AraC is usually given as a continuous infusion at 100-200 mg/m^2 for seven days together with infusion of daunorubicin at 45 mg/m^2 for 30 minutes daily for the first three days (42). Consolidation treatment is initiated after the achievement of complete hematological remission (5% or less of leukemic blasts

in the bone marrow (43)). A commonly used regimen for patients below 60 years of age is high dose AraC (3000 mg/m^2) administered twice daily in 3-hours infusions on days 1, 3 and 5. This consolidation treatment is repeated until a total of 4 consolidations (42). Due to a high risk of treatment-related toxicity for patients above 60 years of age alternative chemotherapy regimen without high dose AraC have to be used for consolidation therapy for elderly patients. However, risk stratification of the patients is important to determine before or early in the consolidation therapy, since allogenic hematopoietic stem cell transplantation (HSCT) is recommended as an alternative consolidation therapy for selected patients (44). Patients younger than 60 years with high risk of relapse can be offered allogenic HSCT if a suitable donor is available. This is reviewed in (45). The final role of autologous bone marrow transplantation in AML remains to be clarified.

These treatment modalities are generally accepted for all AML-patients with the exception of patients with acute promyelocytic leukemia (APL). APL patients have a t(15;17) translocation resulting in the fusion protein PML/RARα and can be efficiently treated with ATRA in combination with chemotherapy (46, 47). Long-term maintenance therapy with ATRA plus low-dose chemotherapy is usually recommended for these patients. Many new treatment options for AML have recently been proposed and tested in clinical trials as discussed in the following section.

Some of the characteristic features of AML are increased proliferation, defects in the apoptosis machinery and increased drug-resistance. These are also the major targets of new therapeutic modalities. The increased proliferation can be targeted by directly inhibiting oncogenic gain-of-function mutations. An example of this is Ras; mutations in *RAS* can be found in 15 – 25% of AML cases (*N-RAS* 20-25% and *K-RAS* 10-15%) (48-53). Anchoring of Ras to the cell membrane via a farnesyl lipid moiety is crucial for Ras activation and thus inhibition of farnesyl trasferase has been suggested as a therapeutic possibility in AML (54). Several clinical trials have demonstrated the antileukemic effect of farnesyl transferase inhibitors (55), although no connection to *RAS* mutations status has been reported. Another example of

targeted therapy in AML is kinase inhibitors specific for the mutated Flt3 receptor *FLT3-ITD*. These activating mutations have been found in 30% of AML patients and represent an adverse prognostic factor. Several small molecule inhibitors directly targeting *FLT3-ITD* are being evaluated in clinical trials. These are excellently reviewed in (56). Table 1 presents an overview of selected new therapy modalities in AML.

Table 1: Selected novel drugable therapeutic targets in acute leukemia

Target molecule	**Drug**	**Tried in leukemia therapy**
DNA	(Alkylators) Cloretazine	(57)
Histone deacetylase§	Valproic acid	(58-61)
	Diphenylbutyrate, Depsipeptide	Reviewed in (62)
DNA methyltransferase§	Decitabine	(63)
Bcl-2§	Oblimersen sodium	(64)
Caspases	CDDO	(65)
Hsp90§	17-allyl-amino-geldanamycin (17-AAG)	Reviewed in (66)
Farnesyl transferase§	R11577 (Tipifarnib, Zarnestra)	(67)
MEK§	U0126	(68)
	PD098059	(69)
Cdks	Cyclin dependent kinase inhibitors	(70, 71)
mTOR§	Rapamycin	Reviewed in (72)
RTK class III:	*Receptor tyrosine kinase inhibitors:*	
Flt3§, c-Kit, FMS, PDGFRα and PDGFRβ	PKC412, CEP-701, CT35318, SU5416 and SU11248	(73-84)
VEGFR	BAY 43-9006, SU11248, SU5416, Vatalanib	Reviewed in (85)
Proteasome§	Bortezomib (Velcade)	(86)
	PR-171	(86)
CD33	Gemtuzumab ozogamicin (Myelotarg)	(87-89)
CD44	Anti CD-44 antibody	Reviewed in (90)
Angiogenesis:		
IL8	IL8 receptor antagonists	Reviewed in (85)
VEGF	Aplidine, Bevacizumab	Reviewed in (85)

Note: § indicate target molecule that putatively influences on the Flt3 signaling pathway.

1.2 Receptor Tyrosine Kinases class III in normal hematopoiesis and leukemogenesis

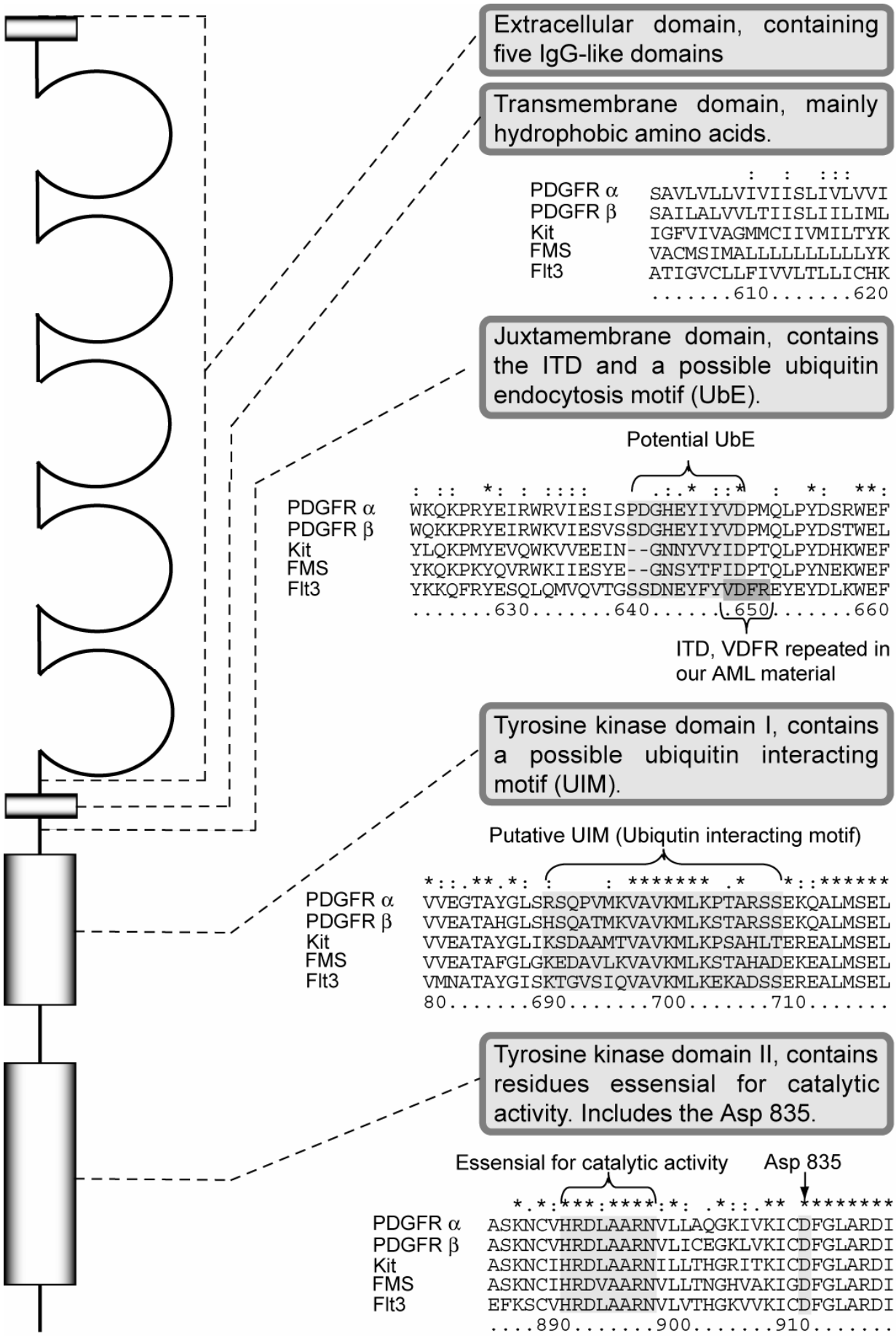
The receptor tyrosine kinases (RTK) class III, also known as the PDGF family, includes the following receptors: c-Kit (91), Fetal-liver tyrosine kinase; Flt3 (92), Platelet-derived growth factor receptor-α; PDGFRα (93), PDGFRβ (94) and c-FMS (95). They are characterized by five immunoglobulin-like domains in the extracellular region, a transmembrane domain, a juxtamembrane (JM) domain and a split tyrosine kinase domain in the intracellular region. An overview of the different domains is showed in **Figure 3**. This group of receptors has important roles in normal hematopoiesis; c-Kit and Flt3 are important for the survival, proliferation and differentiation of early hematopoietic progenitor cells and c-FMS is important for the growth and differentiation of the monocyte-macrophage-osteoclast lineage. In addition, PDGFRβ and its ligand have been suggested a role in megacaryocytopoiesis (96).

The receptors in the RTK class III family can be expressed by primary human AML cells with varying extent. A functional and proliferation-inducing c-Kit receptor is expressed in 60 – 90% of *de novo* AML (97, 98) . Mutations in c-Kit have been reported in AML cells with inv(16) or t(8;16) (13 - 48% overall) and may be associated with an adverse prognosis (99-105). In addition, a fusion protein of PDGFRβ (TEL-PDGFRβ) expressed together with another fusion protein AML1-ETO can induce AML in a mouse model (106). However, the most important receptor in AML is Flt3. Activating mutations in Flt3 are the strongest single predictor for AML relapse after intensive chemotherapy (21), and this will be thoroughly discussed in upcoming sections.

1.3 FMS-like tyrosine kinase 3 (Flt3)

The names FMS-like tyrosine kinase 3, fetal liver kinase 2 and stem cell tyrosine kinase 1 all represent the same protein; the tyrosine kinase receptor Flt3 (92, 107, 108). The *FLT3* gene is located on chromosome 13 (13q12), is over 1000 kilobases long and consists of 24 exons (109-111). The gene encodes a 993 amino acid protein which is detected as two bands of 130 and 160 kilo Daltons (kDa) on Western blots. The 160 kDa band is a result of N-linked glycosylation of the extracellular domain and this is the membrane bound form. The 130 kDa band is unglycosylated and located to the cytoplasm (92, 107, 108, 112, 113).

Figure 3 Overview of the structure of the class III Receptor Tyrosine Kinases. *The sketch and alignments are representative for all RTK class IIIs, while the details in the shaded boxes are specific for Flt3. The line above the alignments is used to mark strongly conserved positions. '*' indicates positions which have a single, fully conserved residue. ':' indicates that one of the following strong groups is fully conserved; STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY and FYW. '.' indicates that one of the following weaker groups is fully conserved; CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM and HFY. The numbers under the alignment indicated residue number of the protein alignment including gaps. All amino acids are represented by their one-letter code.*


Extracellular domain, containing five IgG-like domains
Transmembrane domain, mainly hydrophobic amino acids.
PDGFR α SAVLVLLVIVIISLIVLVVI
PDGFR β SAILALVVLTIISLIILIML
Kit IGFVIVAGMMCIIVMILTYK
FMS VACMSIMALLLLLLLLLLYK
Flt3 ATIGVCLLFIVVLTLLICHK
.....610.....620
Juxtamembrane domain, contains the ITD and a possible ubiquitin endocytosis motif (UbE).
Potential UbE
PDGFR α WKQKPRYEIRWRVIESISPDGHEYIYVDPMQLPYDSRWEF
PDGFR β WQKKPRYEIRWKVIESVSSDGHEYIYVDPMQLPYDSTWEL
Kit YLQKPMYEVQWKVVEEIN--GNNYVYIDPTQLPYDHKWEF
FMS YKQKPKYQVRWKIIESYE--GNSYTFIDPTQLPYNEKWEF
Flt3 YKKQFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEF
.....630.....640.....650.....660
ITD, VDFR repeated in our AML material
Tyrosine kinase domain I, contains a possible ubiquitin interacting motif (UIM).
Putative UIM (Ubiqutin interacting motif)
PDGFR α VVEGTAYGLSRSQPVMKVAVKMLKPTARSSEKQALMSEL
PDGFR β VVEATAHGLSHSQATMKVAVKMLKSTARSSEKQALMSEL
Kit VVEATAYGLIKSDAAMTVAVKMLKPSAHLTEREALMSEL
FMS VVEATAFGLGKEDAVLKVAVKMLKSTAHADEKEALMSEL
Flt3 VMNATAYGISKTGVSIQVAVKMLKEKADSSEREALMSEL
80.....690.....700.....710.....
Tyrosine kinase domain II, contains residues essensial for catalytic activity. Includes the Asp 835.
Essensial for catalytic activity
Asp 835
PDGFR α ASKNCVHRDLAARNVLLAQGKIVKICDFGLARDI
PDGFR β ASKNCVHRDLAARNVLICEGKLVKICDFGLARDI
Kit ASKNCIHRDLAARNILLTHGRITKICDFGLARDI
FMS ASKNCIHRDVAARNVLLTNGHVAKIGDFGLARDI
Flt3 EFKSCVHRDLAARNVLVTHGKVVKICDFGLARDI
....890.....900.....910.....

Flt3 signaling

FL binds to Flt3 as a dimer, this triggers receptor dimerization and *trans*-phosphorylation of tyrosine residues in the JM domain followed by a conformational change (114). This exposes phosphoryl acceptor sites in the tyrosine kinase domain and subsequently causes autophosphorylation and activation of the receptor and the downstream signaling cascade. These intracellular events involve phosphorylation and activation of several cytoplasmic pathways important for the regulation of apoptosis, proliferation and differentiation.

The binding of FL to Flt3 leads to phosphorylation of adaptor proteins like Gab-1, Gab-2 and SHP-2, which in turn bind to and activate the p85 subunit of the phoshoinisitol-3 kinase (PI3K) (115, 116). PI3K is involved in numerous signaling pathways of which Akt signaling is the most important with regards to Flt3. Akt is activated by Flt3 in a FL dependant manner and the downstream transcription factor FOXO3a is inactivated (117). This leads to cell cycle arrest and apoptosis induction. Another important downstream protein of Akt is mTOR (mammalian target of rapamycin). mTOR is aberrantly activated in more than 70% of AML cases and is an important new therapeutic target in AML (reviewed in (72)). The abnormal activation of mTOR leads to increased cell survival of AML cells.

Several components of the Ras/Mitogen activated protein kinase (MAPK) pathway are also phosphorylated upon FL stimulation leading to activation of Ras (118, 119). Ras activates Raf-1 which subsequently leads to phosphorylation and nuclear translocation of Erk-1 and Erk-2. These two mediators catalyze phosphorylation and nuclear translocation of transcription factors and are constitutively phosphorylated in cells with mutated Flt3 (118-121).

The Jak/STAT pathway is also important in Flt3 signaling. Signal transducer and activator of transcription 5; STAT5 has been reported to be constitutively activated in cells with mutated Flt3 (118, 119, 122, 123). Also STAT5 target genes

are induced in these cells, while the wild type receptors show little signaling trough STAT5. An overview of Flt3 signaling is shown in **Figure 4**.

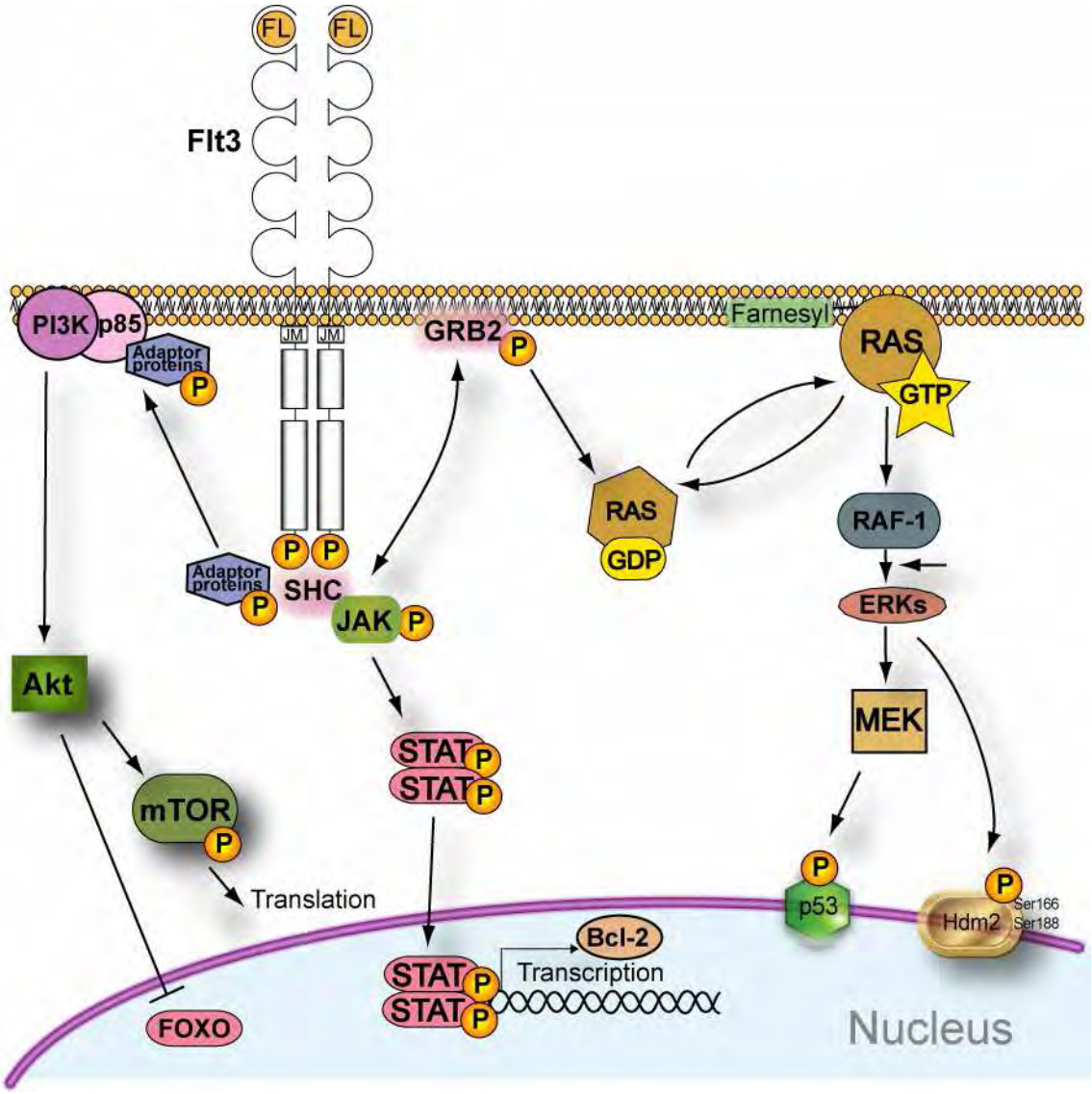


***Figure 4. Flt3 signaling**. The different proteins implicated in Flt3 signaling are described in the text. Activation of the transcription factors STATs lead to the transcription of survival factors like Bcl-2. The Ras/Raf pathway mediates phosphorylation of p53 and Hdm2 and promotes apoptosis and growth arrest.*

Flt3 and normal hematopoiesis

Flt3 is highly expressed in hematopoietic progenitor cells in the bone marrow as well as in thymus, lymph nodes, brain, placenta and liver (92, 124). The expression is usually lost when cells differentiate. The ligand for Flt3 (FL) is almost ubiquitously expressed in either a membrane-bound or a soluble form (125-127). Hence, the function of FL is specified by the restricted expression of Flt3. Most of the information about the role of Flt3 in normal hematopoiesis has come from knock-out studies in mice. Knock-out of either Flt3 or FL leads to viable mice with no hematological diseases and a normal life span (128, 129). However, these mice have reduced number of B progenitor cells, natural killer cells and dendritic cells.

FL regulates early hematopoiesis by stimulating the Flt3 signal transduction pathway, but this is inefficient when FL is present as a single cytokine. The stimulating effect of FL mediated signaling is dependant upon a synergism between FL and several other cytokines (e.g. interleukin-3 (IL-3), granulocyte colony-stimulating factor (G-CSF), colony-stimulating factor-1 (CSF-1) and granulocyte macrophage colony-stimulating factor (GM-CSF)) (113, 130-133).

Flt3 and leukemogenesis; internal tandem duplications and point mutations

Mutations in Flt3 occur in approximately 30% of AML patients. The exact mechanism for the formation of ITDs is unknown, both slippage of the replication machinery and a failure in a mismatch repair mechanism has been proposed (134, 135). There are two major types of Flt3 mutations; in frame internal tandem duplications (ITDs) of 3 – 400 base pairs in the juxtamembrane (JM) region (14, 136) and point mutations of the activation loop in the kinase domain (15, 20). The mutations in the JM domain are mostly internal tandem duplications, but there have also been reports of deletions and insertions in this region. All these mutations are

collectively referred to as length-mutations (LM), but the term ITD is most commonly used and will be used throughout this text.

The juxtamembrane region of Flt3 has been subdivided into three structural domains according to their relative location to the tyrosine kinase domain; the JM-binding motif (JM-B), the JM-switch motif (JM-S) and the zipper or linker peptide segment (JM-Z) (114). When this domain is in its auto-inhibitory state, the JM-B motif makes contact with the most important structures in the kinase domains. The JM-S domain contains two important tyrosine residues (the YΦY motif; where Φ represent a hydrophobic residue) which promote a framework between the JM domains and the kinase domains. Phosphorylation of these tyrosines leads to collapse of the auto-inhibitory state and hence activation of the receptor. ITDs in the JM region lead to disruption of the conformation of JM-B and JM-S and thereby relieve the auto-inhibitory state without phosphorylation of the YΦY motif.

There are reported various mutations in the activation loop of Flt3, all resulting in constitutive active receptor. The most common is missense mutations of aspartate (D) 835 to tyrosine (Y) (15, 20). Mutations of the corresponding amino acid in c-Kit leads to a conformational change in the active loop structure and subsequent constitutive activity of the receptor (for review see (16)). Other activating mutations in the same region have been described; tyrosine (Y) 841 to cystein (C), giving a switch from an open to a closed conformation in the Flt3 activation loop (18) and asparagine (N) 842 to isoleucine (I) which destabilizes the hydrogen-bonding network in the activation loop (17). A destabilizing six base-pair insertion between codons 840 and 842 has also been reported (19).

Flt3 and chemoresistance in human AML

The prevalence of *FLT3* mutations in AML has been reported in numerous studies including more than 5000 individuals (summarized in (137)). The most important conclusions from these studies are as follows:

- *FLT3-ITD* is strongly associated with increased number of leukemic blasts in the peripheral blood and bone marrow of AML patients (136, 138-142)
- *FLT3* mutations are associated with normal or intermediate-risk cytogenetic characteristics e.g. t(15;17). In addition, mutations in *NPM1* are highly associated with *FLT3-ITD* (143).
- *FLT3-ITD* is the strongest separate predictor for disease relapse (21)
- *FLT3-ITD* is an independent poor prognostic factor for overall survival, disease-free survival and event-free survival (137).

Since *FLT3-ITD* is associated with a poor prognosis, a routine screening for *FLT3* mutations is recommended for risk stratification of AML patients. However, the optimal treatment for AML patients with *FLT3-ITD* needs to be further elucidated. A retrospective study from the Medical Research Council of the UK showed that HSCT did not alleviate the adverse effects of *FLT3-ITD* in AML (21).

The precise mechanisms for the increase risk for relapse in AML with FLT3-ITD are currently unknown. Increased DNA-damage repair has been reported to be associated with FLT3-ITD in AML cell lines (144). This may be one contribution to the high risk for relapse in *FLT3-ITD* AML because these cells have enhanced capabilities of surviving DNA-damage therapy. Flt3 is also known to modulate cell adhesion molecules like very late antigen (VLA)-4 (145). This may represent a leukemia-host aspect of chemoresistance and can thus only be studied *in vivo* or in appropriate animal models. Interaction between VLA-4 on AML cells and stromal fibronectin seem to promote minimal residual disease (146).

1.4 The p53/Hdm2 pathway

Human homologue of murine double minute 2 (Hdm2)

The transforming potential of three genes that was over-expressed in a spontaneously transformed BALB/c cell line was connected to small extrachromosomal nuclear bodies called double minutes. These genes were called *mdm1*, *mdm2* and *mdm3* for mouse double minute 1-3. Later, the gene product of *mdm2* was shown to be responsible for the transforming potential of these cells (147, 148). Over-expression of Mdm2 leads to transformation and also to tumor formation in nude mice. The human homologue of Mdm2 is Hdm2. Increased levels of Hdm2 are associated with various cancers e.g. breast cancer, osteosacroma and soft tissue sarcoma. Soon after its discovery, Hdm2 was shown to inhibit p53-induced transformation and it was found that the over-expression of Hdm2 was most prominent in cancers with wild type p53 (149, 150).

Hdm2 in regulation of p53

p53 is a tumor suppressor with anti-proliferative and pro-apoptotic effects on normal cells. It is important to keep the level of p53 low; hence regulation of p53 activity is an important task for the cell. Hdm2 is the key regulator of p53. p53 and Hdm2 form an autoregulatory feedback loop and the two proteins mutually control their levels. Hdm2 is an E3 ubiquitin ligase responsible for the mono-ubiquitination and marking of p53 for degradation (151-154). The further poly-ubiquitination of p53 is mediated through the transcriptional co-activator p300/CBP and this step is dependent on previous mono-ubiquitination by Hdm2 (155). Numerous proteins have been given an important role in the interplay between Hdm2 and p53; among these is the cytoplasmic tyrosine kinase c-Abl. The kinase activity of c-Abl is necessary for the maintenance of basal p53 protein level, and c-Abl increases p53 level upon DNA-damage through prevention of ubiquitination and nuclear export of p53 by Hdm2

(156). The mechanism behind c-Abl induced p53 stabilization is proposed to involve c-Abl phosphorylation of Hdm2 on Tyr394 (157). An overview of selected members in the p53/Hdm2 regulatory pathway is shown in Figure 5.

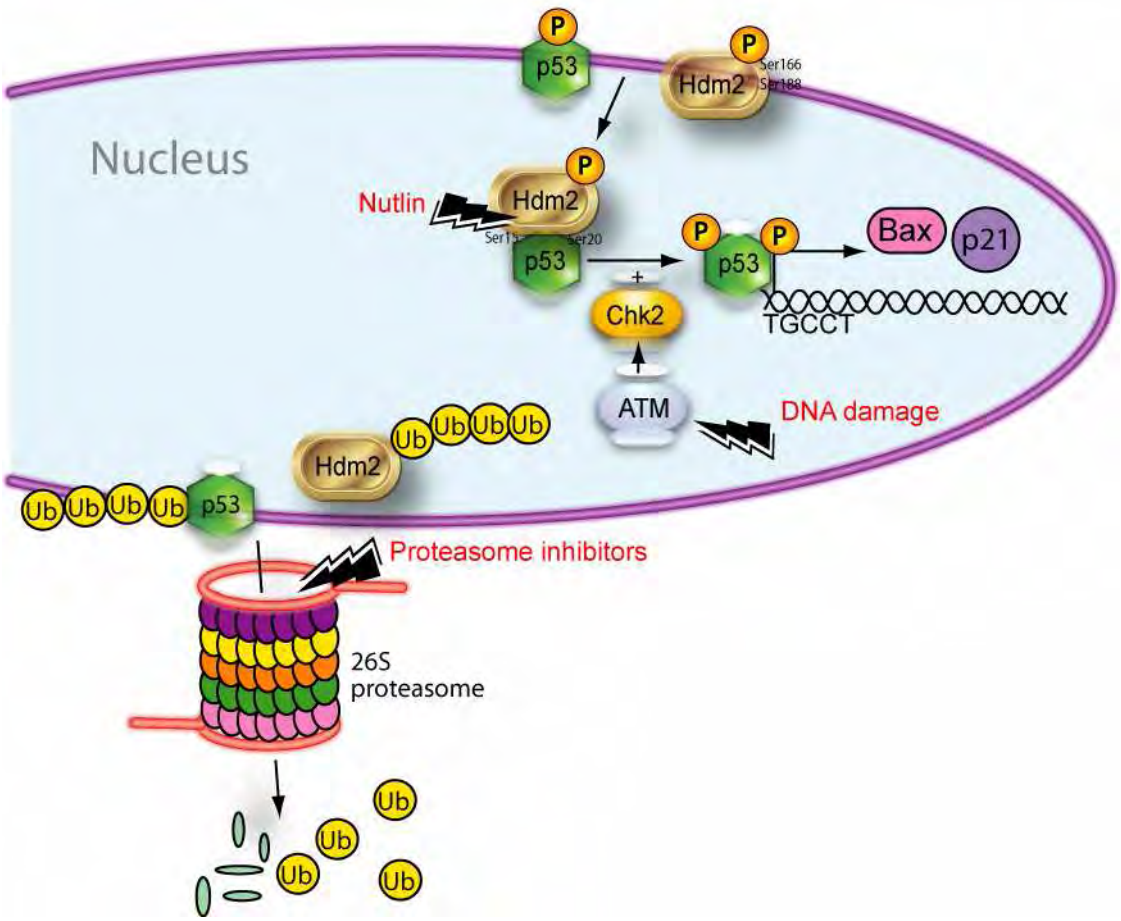


***Figure 5. The p53/Hdm2 regulatory pathway**. p53 is activated upon DNA damage leading to transcription of target genes and subsequent apoptosis, cell cycle arrest and differentiation. Hdm2 binds to and serves as a regulator of p53. The small molecule inhibitors nutlin abrogated the binding of Hdm2 and p53.*

p53 independent functions of Hdm2

Hdm2 does not only function in the regulation of p53. There is evidence for the interaction of Hdm2 with a number of other proteins, including the tumor

suppressor p14ARF (158), the retinoblastoma protein pRb (159), the ribosomal protein L5 (160), E2F/DP1 (161) and the p73 transcription factor (162). These interactions are examples of p53-independent functions of Hdm2, as reviewed in (163, 164). Particularly interesting in view of the topic in this thesis is the role of Hdm2 in ubiquitination and regulation of receptor endocytosis. Hdm2 regulates the trafficking of β2-adrenerg receptor trough ubiquitination of both the receptor and the protein β-arrestin (165). The insulin-like growth factor-1 receptor (IGF-1R) is also ubiquitinated by Hdm2 (166) and this ubiquitination is necessary for the proteasomal degradation of IGF-1R. Hdm2 is also important in the regulation of several hormone binding receptors (i.e. glucocorticoid receptor, estrogen receptor and androgen receptor) (167, 168).

Hdm2 in AML

In contrast to most solid tumors, the *TP53* gene encoding the p53 protein is usually wild-type in AML (169-171). It has been suggested the inactivation of p53 in these cells occurs trough over-expression of Hdm2 since *HDM2* mRNA and Hdm2 protein is over-expressed and has been associated with a poor prognosis in AML (172-176). This over-expression may play an important role in the biology of AML. A small-molecule antagonists of Hdm2 (the isostereomere nutlin-3a) induces p53-dependent apoptosis in AML cells with wild-type p53 and cells with a high Hdm2 level are most susceptible to nutlin-induced apoptosis (172). The molecular mechanism of nutlin-3a is explained through its binding to the p53-binding pocket of Hdm2 and subsequent displacement of p53 from the complex (177). Recently another mechanism for nutlin-3a is described, namely the blockage of Hdm2 binding to E2F1. This results in an enhanced effect of chemotherapy in cells with mutated *TP53* and represents a novel therapeutic approach in these cells (178).

1.5 The ubiquitin-proteasome pathway

Protein degradation and regulation by ubiquitin conjugation

More than 80% of all eukaryotic protein degradation is controlled by the ubiquitin (Ub)-proteasome pathway (179) that includes both polyubiquitination of lysine residues and the following degradation of targeted protein by the proteasome. The proteasome is a large intracellular protease with multiple enzyme activities consisting of a 20S catalytic core complex and two 19S regulatory subunits (reviewed in (180)). The ubiquitination process is mediated trough the three enzyme families E1, E2 and E3. E1 is an Ub-activating enzyme which binds to and activates Ub. The next step in the ubiquitination process occurs when an Ub-conjugating E2 enzyme transfers an activated Ub to an E3 Ub-ligase that attaches an Ub to the desired protein. These steps are repeated until an Ub-chain is formed (reviewed in (181)). The ubiquitinated protein binds to the 19S subunit of the proteasome where it is de-ubiquitinated and unfolded. The unfolded protein is transported to the 20S subunit and degraded into peptides of various lengths (reviewed in (182)).

Polybiquitination is mostly associated with the degradation of target proteins, while monoubiquitination has been described as a regulatory signal, similar to post-translational modifications like neddylation, sumoylation, acetylation and phosphorylation. Monoubiquitination is involved in the regulation of histone function (183), membrane trafficking (reviewed in (184)), transcription regulation (185), DNA repair (186, 187) and DNA replication (188). Receptor tyrosine kinases undergo ligand-dependent ubiquitination and this has become recognized as an important signal for endocytosis and degradation in the lysosome. It has been shown that epidermal growth factor receptor (EGFR) and PDGFR are not polyubiquitinated but monoubiquitinated at multiple sites after ligand-induced activation and that monoubiquitination is the principal signal responsible for the movement of RTKs from the plasma membrane to the lysosome (189).

Of particular interest for the work presented in this thesis is the fact that the proteasome regulates short lived proteins as the tumor suppressor p53 (190) and the anti-apoptotic protein Mcl-1 (191). Proteins with much longer half life are also degraded via the proteasome, including the anti-apoptotic protein Bcl-2. The spatio-temporal regulation of ubiquitination seems to be determined by the E3 ligase activity.

E3 ubiquitin ligases

The E3 ubiquitin ligase family consists of around 1000 proteins that can be divided into three major types based on structure and substrate recognition. The first class is N-end rule ubiquitin ligases which target proteins with specific destabilizing N-terminal residues (192). The second group consists of HECT-domain (Homologous to the E6-AP Carboxyl Terminus) E3 ligases and the third and largest group is the RING (Really Interesting New Gene) family of E3 ligases. There have also been reports of E3 ligases with no similarity to any of the groups, linking the E3 ligase activity to new protein sequences. E3 ubiquitin ligases have also been indicated as both targets for cancer therapy and as biomarkers. This is reviewed in (193).

Examples of E3 ubiquitin ligases are the RING-finger ubiquitin ligase; Hdm2 (see above), the Cbl RING-finger ubiquitin ligases and the p300-CBP-associated factor (PCAF), a newly described atypical E3 ubiquitin ligase. The Cbl family of ubiquitin ligases plays a major role in the ligand-dependent ubiquitination of many receptor tyrosine kinases (194). Cbl binds directly to c-Kit (195), and also to Flt3 (196). An inactivating mutation in c-Cbl was recently found in the bone marrow of one AML patient, this mutation lead to Flt3 dependent transformation of AML cells *in vivo* (196). PCAF is a histone acetyltransferase involved in the regulation of p53 and it possesses an intrinsic ubiquitin ligase activity which is critical for the control of Hdm2 expression levels (197). This ubiquitin ligase activity does not map to any known E3 ubiquitin ligase motif.

Functional role in AML

The ubiquitin-proteasome pathway has been validated as an important therapeutic target in several hematological malignancies (198). Proteasome inhibitors are also suggested as a group of new therapeutic agents for the treatment of AML. The proteasome inhibitors bortezomib and PR-171 has been tested *in vitro* with promising effects on primary AML cells (86). The importance of the E3 ligases Hdm2 and Cbl in AML can also lead to speculations of the possible targeting of the ubiquitin system in AML.

1.6 The Bcl-2 family of proteins

Overview

Members of the Bcl-2 family of proteins are key regulators of apoptosis and at least 19 different family members have been identified in mammalian cells (reviewed in (199, 200)). These proteins are divided into three groups; 1) the anti-apoptotic Bcl-2 subfamily (Bcl-2, Bcl-X_L, Bcl-w, Mcl-1 and A1/Bfl-1), 2) the pro-apoptotic Bax subfamily (Bax, Bak and Bok/Mtd) and 3) the more distant relatives, the pro-apoptotic BH3-only proteins (Bik/Nbk, Blk, Hrk/DP5, BNIP3, Bim_L/Bod, Bad, MAP-1, Bmf, Noxa, Puma/Bbc3 and Bid). The pro-apoptotic Bcl-2 proteins localize to the mitochondria where they control the permeabilization of the mitochondrial outer membrane and subsequent cytochrome *c* release to the cytosol and further caspase activation. The exact mechanisms for regulation of the Bcl-2 proteins involve numerous modifications and different factors not completely elucidated (reviewed in (200)). Recently, p53 has been shown to bind directly to Bcl-2 and thereby disrupt the Bcl-2/Bax binding and thus enhance Bax-dependent apoptosis (201). Conversely, phosphorylation of Bcl-2 in a positive regulatory region abrogates Bcl-2/p53 binding and favors cell survival.

B-cell lymphoma gene 2 (Bcl-2)

Bcl-2 is the prototype for this family of proteins and belongs to the anti-apoptotic subfamily. The name B-cell lymphoma-2 is given since Bcl-2 is the second gene from a range of proteins over-expressed in follicular lymphoma. The human *BCL-2* gene is located at 18q24 on the long arm of chromosome 18. Bcl-2 has been implicated in a number of cancers, e.g. melanoma, breast cancer, prostate cancer and lung cancer and is thought to be involved in resistance to conventional cancer treatment.

Myeloid cell leukemia 1 (Mcl-1)

Mcl-1 is an anti-apoptotic member of the Bcl-2 family of protein, identified in 1993 in differentiating myeloid cells (202). Mcl-1 contains three Bcl-2 homology (BH) domains and a C-terminal transmembrane domain which localizes Mcl-1 to the mitochondrial membrane. N-terminally, Mcl-1 contains two PEST domains. This is characteristic of proteins with a short half-life; Mcl-1 has a half-life of one to a few hours (203-205). An elimination of Mcl-1 from the cell is a necessary step in the induction of mitochondrial apoptosis; therefore Mcl-1 is rapidly degraded following DNA-damage (205). Mcl-1 has also been shown to have an important role in the regulation of p53 induced apoptosis. p53 and Mcl-1 both interact with and modulate the activity of the death effector Bak and thereby have opposing effects on mitochondrial apoptosis (206).

Bcl-2 family members in AML

Bcl-2 over-expression is associated with prolonged survival of malignant cells and chemoresistance in AML (207). The ratio of Bax to Bcl-2 has been proposed as a prognostic indicator in AML (208). A high Bax/Bcl-2 ratio is associated with a

higher complete remission rate and a longer overall survival. Inhibition of both Bcl-2 and Hdm2 with small-molecule inhibitors leads to the synergistic induction of apoptosis in both AML cell lines and primary cells (209). It has also recently been shown that high levels of Bcl-2 are associated with p53 hyper-phosphorylation and *FLT3-ITD* in AML (210).

2. Aims of the study

The aim of this thesis has been to elucidate the differences between Flt3-wt receptor and Flt3-ITD receptor in AML, with particular focus on the following topics:

1. the role of Flt3 and Flt3 signaling in primary AML cells *in vitro*
2. the modulation of Flt3 and *FLT3-ITD* during DNA damage therapy.
3. Flt3 signaling and its modulation of the p53/Hdm2/Bcl-2 pathway.

3. Methodological considerations

Several different methods and techniques have been utilized in the work of this thesis. The use of primary cells from a biobank of consecutive AML patients admitted to Haukeland University Hospital has been a major advantage. However, this cell material is limited, and the use of more experimental systems, like AML cell lines, had to be considered. Determination of designated protein levels, either static or in treatment response experiments has been a major part of this thesis. Various methods have been evaluated, the results of which are discussed below. In addition to static protein levels, the interplay between proteins in the cell is important. There are different methods to determine protein-protein interactions, some of which will be discussed below.

3.1 Choice of experimental cell model

AML cell lines versus primary AML cells

In paper I, primary AML cells were utilized in a well established setup to study proliferation, cytokine secretion and apoptosis. These methods are all set up in a 96- and 24-well format requiring only a minimum of cells. In this paper we also established Western blots as a method to quantify protein expression and this method was used throughout papers II to IV. This is discussed in more detail below. As Western blots are a more material demanding method, we chose to use AML cell lines as our main source of material in the next papers.

Previous works from our group indicate that the use of cell lines has obvious limitations in reflecting modulation of important *in vivo* cell signaling pathways (123, 211). Others have pointed out that the gene expression profiles of breast cancer cell lines are quite distant from profiles from primary breast tissue (212). Investigation of

DNA-damaging therapy has recently elucidated cancer-host reactions as important to understand the complete effect of chemotherapy in animal models (213). This illustrates yet another limitation when using cell lines in *in vitro* experiments.

Most AML cell lines comprise mutations in *TP53* as this is part of the immortalization process. Primary AML cells, in contrast, rarely have *TP53*-mutations.(169-171). *In vitro* both cell lines and primary cells are easy to kill with chemotherapeutics, while patients harboring mutated *TP53* in their AML cells have a notoriously bad prognosis (36). Together, this implies that cell lines and primary cells should be used complimentary, and that the *in vivo* situation needs to be taken into consideration.

3.2 Protein quantification

Quantification in Western blots and by flow cytometry

In paper I we investigated the relationship between Flt3 protein level and Flt3 mutational status, but found no correlation. The protein levels were determined by Western blots and quantification of signal intensities with Kodak 1D software from cell lysates stored in -80ºC. A 70 kDa band from Jurkat cells was used as intragel control. The lack of correlation is most likely due to inaccuracy in the quantification and also to the lack of protein detection for many of the samples. The cause of this can be degradation of protein samples during harvest, preparation and handling. The use of a cell line as intra-experimental control was also an issue we reconsidered when retying to establish a correlation in later experiments. In paper III we utilized cryopreserved primary AML cells as source for determination of Flt3 protein level. Because of the problem with protein degradation in cell lysates, we chose to use flow cytometry as a means for determining Flt3 and Hdm2 protein levels in paper III. Even if protease activity may happen in the flow cytometric procedure, viable cells or

swiftly fixated cells were analyzed immediately, securing a limited degradation. Normal peripheral blood mononuclear cells (PBMCs) from the same donors were used as intra-experimental controls. Both surface Flt3 determined by extracellular flow cytometry and total Flt3 determined by intracellular flow cytometry showed a strong correlation to Flt3 mutational status with highest level in Flt3-ITD patients.

Variance by flow cytometry was less than the variance observed when using Western blots, but Western blots have its advantage when information about molecular weight is needed. In some cases a simple Western blot may provide the researcher with important information about the target protein based on small changes in molecular weight, e.g. phosphorylation and glycosylation. However, these modifications can be more specifically examined by flow cytometry if verified modification-specific antibodies are used.

An advantage of single cell analysis like flow cytometry is that is allows studies of distinct subsets of cells. This is not approachable when using cell lysates (214). Flow cytometry can also be used for the quantification of receptor number on the cell surface. Commercial kits are available that uses microbeads conjugated with defined number of antibodies to establish calibration curves for the determination of receptor number on the cell surface (215).

Intra- and extracellular flow cytometry

Extracellular flow cytometry is a well-known technique in hematology, used to determine surface markers (CD markers; CD denotes cluster of differentiation) on blood cells. Since Flt3 is a surface protein (CD135) we have used this technique to determine surface Flt3. However, since the cellular pool of Flt3 protein also includes an intracellular part, we decided to investigate the Flt3 level in permeabilized cells as well. Intracellular flow cytometry is a powerful technique that allows simultaneous

analysis of up to 17 parameters (216). The advantages of this technique compared to e.g. ELISA or Western blotting are many (reviewed in (217)).

In paper III, the levels of total Flt3 was only slightly higher than levels of surface Flt3 in the primary AML cells. Like Western blot determination of Flt3, flow cytometric determination of total Flt3 should be the sum of surface Flt3 and intracellular Flt3. However, we only observed a minor augmentation when comparing the mean values from surface and total staining. This difference may reflect the technical difference between the two methods. When staining for surface protein, the cells are live and only fixated after the fluorescent antibody is bound. The intracellular staining requires a permeabilization step. The cells are therefore fixed in paraformaldehyde prior to the permeabilization in methanol; the cells are then stained with fluorescent antibodies after the permeabilization step. The quality of the antibodies is also very important in flow cytometry applications. Visualization of IR-induced Hdm2 down-regulation was not possible using flow cytometry, but was easy when using Western blots. The same clone of antibody was tried on both flow cytometry and Western blots, but only the Western blots showed the down-regulation. A reason for this inconsistency may be that the epitope for the antibody unavailable when preparing the samples for flow cytometry.

The advantages of intracellular flow cytometry are many; the use of modification-specific antibodies is mentioned before. When comparing the levels of both surface and intracellular proteins in the same experimental setup, the use of one standardized staining protocol is recommended. The comparison of values obtained from different protocols is difficult and requires the use of carefully designed control samples.

3.3 Immunoprecipitation

Both paper III and IV raises questions about protein-protein interaction as a part of the regulation of Bcl-2, Hdm2 and Flt3. Co-immunoprecipitation is a natural choice of technique to illustrate native protein-protein interactions and has been utilized in this thesis as an approach to detect interactions between central players in the Flt3/p53/Hdm2/Bcl-2 pathways. There are also several other approaches to illustrate protein-protein interaction; e.g the Yeast Two Hybrid System or FRET (Fluorescence resonance energy transfer). However, these methods require an over-expression of the proteins of interest, and might not represent the normal situation in the cell.

Co-immunoprecipitation; determination of protein-protein interactions

In spite of numerous attempts to demonstrate an interaction between endogenous Flt3 and Hdm2, and also between Hdm2 and Bcl-2, no such interactions were found by co-immunoprecipitation. Several different immunoprecipitation systems have been utilized in these attempts. We have tried assays where the antibody, cell lysate and capture resin were in the same solution, assays where the antibody was conjugated to a semisolid gel and systems utilizing small magnetic particles to capture the antibody/protein complex. All these different methods had their advantages and disadvantages, but in our hands they only worked in immunoprecipitation. None of the desired co-immunoprecipitations was convincingly and reproducibly performed, but these negative results do not mean that no interactions exist.

One challenge with immunoprecipitations is the high levels of immunoblobulins from the antibody that may contaminate the Western blots. The heavy and light chains of immunoglobulins give bands at approximately 25 and 50 kDa. However, incomplete disruption of immunoglobulins may give reactivity on the

Western blots above these molecular weights. A way of avoiding this is to use antibodies for Western from other species. This is more easily done for well studied proteins where the number of commercially available antibodies is high. The choice of buffer is also important. A native protein-protein interaction can be very fragile, so the buffer needs to mimic the intracellular or subcellular condition for the particular interaction (218).

For future work, epitope tagged proteins and over-expression could be tried to elucidate putative interactions between Flt3 and Hdm2 and between Hdm2 and Bcl-2, in addition to novel flow cytometry-based techniques.

Alternatives to co-immunoprecipitation

A classical method of detecting protein-protein interactions is the yeast two-hybrid (Y2H) system (219, 220). A problem with the Y2H-system is that is requires a nuclear localization of the proteins in interest. In addition, the proteins need to fold correctly in the nucleus. This can be an obstacle for membrane proteins like Flt3. A novel system has been designed for the study of interactions between membrane proteins and cytoplasmic proteins; namely the split-ubiquitin system where interactions between protein X and Y can be detected by monitoring the size of the reporter proteins on Western blots (221). Modified versions of this system have been successfully used to screen for interactions associated with membrane proteins (222, 223).

Fluorescence (Förster) resonance energy transfer (FRET) is also a very powerful technique for the detection of protein-protein interactions in living cells. FRET is based on a transfer of energy between two fluorescent molecules; a donor molecule which is exited with subsequent energy transfer to an acceptor molecule. This causes an emmition of fluorescence from the acceptor (224). A new method combining FRET with the localization of target proteins on the cytoplasmatic surface

of the plasma membrane has recently been described (225). This method and other very useful techniques for the study of protein-protein interactions is nicely reviewed in (226).

4. Summary of papers

4.1 Paper I

FLT3-ITD is a frequent mutation in AML, and is associated with recurrent relapse and poor prognosis after chemotherapy. Mutated Flt3 is constitutively active, while Flt3-wt is activated upon binding of its ligand (FL). We wanted to investigate the effects of exogenous FL on primary AML cells with and without Flt3 mutations *in vitro*. Our first observation was that the basal protein level of Flt3 varied among the patients and did not correlate to Flt3 mutational status as judged by Western blot in 42 patients examined. Further, the secretion of FL, AML blast colony-formation, cytokine secretion, morphology and apoptosis induction varied among the AML patients and was not influenced neither by the addition of FL nor by the Flt3 mutational status of the patients. However, the majority of AML blasts showed an increased proliferation upon FL stimulation both alone and in combination with various cytokines. The increased proliferation was not significantly different in AML cells with mutated versus wild-type Flt3. This suggests that signaling through Flt3 occurs irrespectively of Flt3 mutational status.

4.2 Paper II

AML cells treated with intensive chemotherapy *in vivo* show a rapid activation of p53 and an induction of p53 target genes. Hdm2 is in addition to its p53 antagonism implicated a role in receptor tyrosine kinase regulation. Thus, we wanted to investigate the effects of therapeutic DNA-damage on the protein and mRNA levels of Flt3, the p53 regulator Hdm2 and Bcl-2 protein family members in relation to Flt3 mutational status and induction of apoptosis. Ionizing radiation (IR) induced concerted regulation of Hdm2 and Flt3 in Flt3-wt cells accompanied by apoptosis and

down-regulated Mcl-1. The IR-resistant *FLT3-ITD* cells also had a response on Hdm2 in spite of unchanged Flt3 and Mcl-1 protein levels. Attenuation of Hdm2 and Mcl-1 was also seen in cells without p53. Another DNA-damaging agent, the anthracycline daunorubicin also induced attenuation of Hdm2 and Mcl-1 independent of *TP53* and *FLT3* status. A reciprocal regulation of Flt3 and Hdm2 was furthermore seen in AML patients undergoing chemotherapy *in vivo*. Based on these findings targeting of Hdm2 and Mcl-1 can be important for optimizing therapy for AML patients with *FLT3-ITD*.

4.3 Paper III

The establishment of a relationship between Flt3 and Hdm2 in paper II called for a more thorough investigation of the reciprocal protein modulations of Flt3 and Hdm2 in relation to Flt3 mutational status. In a flow cytometry assay, the level of Flt3 was significantly higher in AML patients with Flt3-ITD than in patients with Flt3-wt. A sequence alignment of Flt3-ITD sequences revealed a potential ubiquitin dependent endocytosis motif in proximity to the duplicated region in Flt3. Further examination showed that Flt3-ITD cells were more rapidly recycled than the Flt3-wt cells. Long term treatment with Flt3 ligand (FL), down-regulation of Flt3 with siRNAs and the kinase inhibitor PKC412 all induced a simultaneous increase in Hdm2 and decrease in Flt3. This was most evident in Flt3-wt cells. However, the use of nutlin-3, an inhibitor of the p53/Hdm2 interaction had no effect on Flt3. These observations suggest a dysregulated Flt3-receptor turnover in *FLT3-ITD* cells in combination with an attenuated Hdm2 down-regulation and tightly connect Flt3 to the p53/Hdm2 pathway.

4.4 Paper IV

Paper III suggested a regulatory interconnection between Flt3 and p53 through Hdm2. A recent paper from our group demonstrated a correlation between Bcl-2, mutated Flt3 and hyper-phosphorylated p53 in AML (211). It was therefore of interest to determine if Bcl-2 could contribute more directly to Hdm2 regulation. A positive correlation between Hdm2 and Bcl-2 levels was seen in various cell lines. This could be manipulated with transient transfections of Bcl-2, over-expression of Bcl-2 and Bcl-2 inhibition by retroviral shRNA expression and HA14-1. Over-expression of Bcl-2 protected cells from IR-induced Hdm2 down-regulation. The expression of Hdm2 with impaired ubiquitin ligase activity led to an increased level of Bcl-2 protein, but had no effect on the IR-induced Hdm2 down-regulation. However, inhibition of E1 ubiquitin activation enzyme activity, inhibition of the proteasome and blocking of poly-ubiquitination by mutant ubiquitin lead to blockage of IR-induced Hdm2 attenuation but had no effect on Bcl-2. These results propose of a novel mechanism for Bcl-2 inhibition of p53-induced apoptosis through Hdm2.

5. General discussion

Bcl-2, Hdm2 and p53 proteins are examined in papers I-IV of this thesis, and will therefore be discussed in the light of current knowledge of Flt3 in AML. The putative involvement of an ubiquitin dependent endocytosis motif in the juxtamembrane region of Flt3 is of particular interest, and this region will be more extensively debated. Finally, the role of *FLT3-ITD* in regulation of the p53/Hdm2/Bcl-2 pathway will be emphasized.

5.1 The juxtamembrane region of Flt3

Sequence similarity of the RTK III family

The multiple sequence alignments in paper III were performed using the ClustalX software described in (227). This software implements a progressive alignment method in which the most similar sequences are aligned first and then less related sequences are added to the alignment until all the sequences have been incorporated. Other methods for multiple sequence alignments include dynamic programming (228), iterative methods (229) and motif finding (e.g. MOTIF (230)). In addition several techniques inspired by computer science have been applied to the multiple sequence alignment problem, the most well-known is hidden Markov models (231). Multiple sequence alignment methods are reviewed in (232). For the use in this paper we believe that ClustalX is satisfactory for recognition of the UbE-domain.

There are high sequence similarities between the five receptors in the RTK class III family; alignments of the juxtamembrane region show 19% sequence identity and 60% sequence homology (data not shown). This sequence homology allows for speculations about the high frequencies of *FLT3-ITDs* in AML, while

related receptors like Kit and PDGFRβ are only rarely affected in AML Internal tandem duplication in itself seems to be a rare mutational occurrence in disease, mostly associated with Flt3 and AML in humans. Up to date there is only a few other reports of ITDs in human diseases, e.g. CBP in esophageal squamous cell carcinoma (233), MLL in AML (234) and in collagene genes associated with spondyloepiphyseal dysplasia and also lethal osteogeneis imperfecta (235, 236). The importance of the juxamembrane region of Flt3 in AML is therefore indisputable and will be discussed in detail below.

Importance of the juxtamembrane region

Several residues in the juxtamembrane region of the RTK class IIIs are shown to have important roles in cancer cell transformation, endocytosis of receptors, as phosphorylation cites and as hot-spots for mutations. These are overviewed inTable 2.

Table 2 Overview of juxtamembrane residues and their function in Flt3

Residue in Flt3	Corresponding in other RTK class III	Importance	Reference
Y572 L576 M578 V579 Y589§ Y591§ Y597 Y599	PDGFR β; Y562 PDGFR β; W566 PDGFR β; V568 PDGFR β; I569 PDGFR β; Y579 PDGFR β; Y581 PDGFR β; L587 PDGFR β; Y589	Residues which cause constitutive activation when mutated in PDGFRβ	(237)
V579 and V592§		Point mutations in Flt3 identified in AML-patients: valine substituted to alanine	(238)
Y589§	PDGFR β; Y579	Critical for ligand mediated endocytosis in PDGFR β	(239)
Y589§ and Y591§		Candidate STAT5 docking sites	(240)
Y589§ V592§	c-Kit; Y568 c-Kit; I571	Phosphorylation of Y568 and presence of I571 essential for binding of Cbl to c-Kit	(195)
Y589§ and Y599		Ligand-induced auto-phosphorylation sites; bind Src family kinases and protein tyrosine phosphatase SHP2.	(241)
Y591§		Duplicated in some patients with Flt3-ITD; have elevated Bcl-2 and potentiated STAT5 signaling	(211)
R595		Duplicated in 77% of Flt3-ITD. Critical for the transforming potential of Flt3.	(242)

Note: § denotes residues included in potential UbE in Flt3

In paper III we hypothesize an ubiquitin dependent endocytosis (UbE) motif in Flt3, in close proximity to the area being duplicated in *FLT3-ITD* patients. Table 2 lists the most important residues in the JM-region of RTK class III and of the ten residues reported as being important in the literature, three are included in the putative UbE motif. In addition, 37% of the AML patients with sequenced *FLT3-ITD* in our collection of patient material have a totally duplicated UbE motif. The consequences of this duplication have not been tested experimentally, but the

possible implications for Flt3 receptor recycling and Flt3 signaling will be discussed below.

Duplicated ubiquitin dependent endocytosis motif (UbE)

The ubiquitin dependent endocytosis motif (UbE) was first described in Growth Hormone Receptor (GHR) by Govers et. al. in 1999 (243). This domain contains 10 amino acids, namely DSWVEFIELD. UbE has been shown to be essential for the internalization of GHR. However, conjugation of ubiquitin to lysine residues in the cytosolic domain of GHR is not required. The sensitivity of cells towards GH is mainly regulated trough the UbE in GHR (244) and this UbE has recently been associated with the ubiquitin ligase SKP1-CUL1-βTrCP complex (245).

The presence of an UbE motif has so far only been studied in GHR, however sequence alignments have revealed similar motifs in other proteins including PDGFR. We describe that most internal tandem duplications of Flt3 are in close proximity to an UbE motif, and that this UbE motif is duplicated in a subset of the sequenced *FLT3-ITD* patients. The implications of a duplicated UbE motif can only be speculated so far. FLT3-ITD has been shown to have a higher Flt3 level and a more rapid receptor recycling. A duplicated UbE motif could result in an even higher Flt3 level and more rapid recycling. An interesting observation is that our patients with *FLT3-ITD* including a duplicated Y591 are shown to have increased Bcl-2 protein level and elevated expression of hyper-phosphorylated p53 (211): These patients actually have the entire UbE motif duplicated (patients # 3, 6, 8 and 14 in Fig. 1, paper III is identical to patients P28, P11, P12 and P05 in (211)). This linkage can guide us towards a functional understanding of the importance of the UbE motif in *FLT3-ITD*.

5.2 *FLT3-ITD* function in chemoresistance and disease relapse

GM-CSF/STAT5 signaling depending on FLT3 mutational status.

In paper I, neither the addition of FL nor the Flt3 mutational status was statistically correlated with the properties examined. However, *FLT3-ITD* is hyper responsive regarding STAT-5 activation after GM-CSF stimulation (123). The mechanism of potentiated GM-CSF signaling in the presence of *FLT3-ITD* has so far not been described. The plasma membrane is not a homogenous surface, but consists of numerous lipid microdomains floating around in the semi-solid membrane; called "lipid rafts". These structures vary in size, composition and stability and have been implicated in signal transduction trough a number of cancer-related pathways (for review see (246)). It is tempting to speculate that *FLT3-ITD* receptors somehow can be retained in such rafts and thereby have a different micromilieu than their wild type counterparts. If *FLT3-ITD* receptor is trapped in lipid rafts with the receptors for GM-CSF, it is not unlikely that these cells will have potentiated GM-CSF signaling.

FLT3-ITD in cell-cell interaction involved in relapse predisposition and chemoresistance?

The leukemia-host interaction is an important aspect of AML. Flt3 has been associated with VLA-4 and VLA-5 via Flt3 ligand (145). This has implications for the homing of Flt3 expressing immature hematopoietic progenitor cells to the bone marrow microenvironment. Several reports indicate that the threshold for chemotherapy-induced apoptosis is elevated in AML blasts that interact with stromal cells (247-249). High expression levels of Flt3 will keep these blasts in the bone marrow, and might contribute to relapse predisposition. The properties of *FLT3-ITD* can hypothetically be associated with increased adhesiveness in the bone marrow, and therefore lead to increased chemoresistance.

5.3 Interaction between Flt3 and Hdm2

In paper II and III, a reciprocal protein regulation of Flt3 and Hdm2 is described, but no convincing direct interaction has been observed so far. However, this does not rule out the possibility for a direct or indirect interaction between Flt3 and Hdm2. The lack of Flt3 response to a nutlin-3 induced up-regulation of Hdm2 has led us to hypothesize that nutlin-3 abrogates an interaction between Flt3 and Hdm2. It is well known that nutlin-3 binds to Hdm2 in the p53 binding pocket and blocks the binding of p53 to Hdm2 (177). In addition it has been shown that nutlin-3 abrogates binding of Hdm2 and Hypoxia-inducible factor-1α (HIF-1α) (250). A putative Hdm2 binding motif in HIF-1α was found in the NH_2 terminus, this motif have sequence similarities with Hdm2-binding motifs in p53, p73 and E2F. The possibility of a similar sequence in Flt3 is speculative but a sequence alignment revealed a vague similarity of these Hdm2-binding domains to a sequence in the extracellular part of Flt3 (Figure 6).

```
                    .           :
p73         --------STATSPDGGTTFEHLWSS-----LEPD------
p53         --------PSVEPPLSQETFSGLWK------LLPEW-----
E2F         --------ADSLLEHVREDFSGLPEE-----FISL------
Flt3/460-500 PSWTWKKCSDKSPNCTEEITEGVWNRKANRKVFGQWVSSST
HIF1a       --------ARSRRSKESEVFYELAHL-----PLP-------
```

Figure 6 Putative nutlin-3 sensitive Hdm2-binding motif in Flt3

The presence of such an Hdm2-binding motif in the extracellular part of Flt3 does not preclude the possibility for an Hdm2-Flt3 interaction. Little is known about the structure of intracellular Flt3 and it is possible to envision a scenario in which the extracellular part of the protein is accessible for Hdm2 binding at some point in the life-cycle of Flt3. As discussed before; several novel techniques are now available for the more thorough investigation of a possible Hdm2-Flt3 interaction and these will be attempted in future experiments.

5.4 Flt3-Hdm2-Bcl-2: How to tightly control wild type p53 in AML

In AML 90% of the patients comprise wild type *TP53*. In this thesis several novel factors have been included as means to control this protein in the AML-cells. When the Flt3 level is decreased, an increase in Hdm2 is seen. Hdm2 is the most important regulator of p53, therefore inhibiting Flt3 will sequentially lead to decreased p53. Hdm2 is also important in the regulation of Bcl-2 and has been shown to be crucial for the most effective Bcl-2 protection against p53-induced apoptosis.

p53-induced apoptosis is important as a means to eradicate cancer cells. Bcl-2 protection against p53-induced apoptosis and also the novel Hdm2 involvement is thus unwanted in the AML cells. Inhibition of Bcl-2 is therefore an alternative to drive the cells towards p53-induced apoptosis.

Based on the evidence shown in this thesis, we postulate a p53/Hdm2/Bcl-2 pathway which is highly modulated by Flt3 in AML. Flt3 and Hdm2 are reciprocally regulated, but the exact mechanisms for this regulation need to be further evaluated. Several mutants of Flt3 and Hdm2 have been made in our lab including Flt3 with a deficient UbE motif, Flt3 with impaired kinase activity, Hdm2 without E3 ligase activity and Hdm2 that cannot be tyrosine phosphorylated. These constructs in addition to *FLT3-ITD* constructs with sequences derived from our patient material will be used to evaluate the mechanisms behind the reciprocal regulation and differences in Flt3 recycling.

Both Bcl-2 and Mcl-1 was regulated by Hdm2 in a positive manner. A persistent Hdm2 and Mcl-1 down-regulation was needed for apoptosis to occur. Bcl-2 protects cells from p53-induced apoptosis; this protection was most effective in the presence of Hdm2. We have made an AML-cell line with Bcl-2 shRNA to further study this regulation. The cell line we have created has wt *TP53* and mutated *FLT3*, a perfect tool for studying the p53/Hdm2/Bcl-2 pathway and modulations by Flt3.

To conclude, the work in this thesis has shown that different signaling pathways in AML are tightly connected. Hdm2 is implicated both a p53-independent role and a role in the p53/Hdm2/Bcl-2 pathway. The differences between wild type Flt3 cells and cells with *FLT3-ITD* regarding the p53/Hdm2/Bcl-2 pathway are obvious and open for novel thinking with regards to therapy. The combined targeting of *FLT3-ITD*, the p53/Hdm2 interaction and Bcl-2 is both speculative and interesting.

6. Future perspectives

The definition of a p53/Hdm2/Bcl-2 pathway opens for interesting studies in AML. More mutagenesis studies need to be performed in order to elucidate the exact molecular mechanisms behind the different regulations and modulations shown in this thesis. An IL3 dependant mouse leukemia cell line (BAF/3) is relatively easy to transfect and gains IL3-independence upon *FLT3-ITD* transfection. This system will be used to further study the mechanisms underlying the novel pathway postulated in this thesis.

Different facets of combination therapy are interesting aspects that need to be tested. The combined targeting of *FLT3-ITD*, the p53-Hdm2 interaction and Bcl-2 can be tested using the inhibitors PKC412, nutlin-3 and HA14-1 in AML-cell lines and patient cells *in vitro*. Furthermore a novel fluorescent reporter system for small animal optical imaging in AML has recently been developed in our lab (McCormack et. al, unpublished data). Animal testing followed by optical imaging analyses are currently being planned.

7. References

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Paper I

Flt3-mediated signaling in human acute myelogenous leukemia (AML) blasts: a functional characterization of the effects of Flt3-ligand in AML cell populations with and without genetic Flt3 abnormalities

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Background and Objectives. Intracellular signaling initiated via Flt3 seems important in both leukemogenesis and chemosensitivity in acute myelogenous leukemia (AML). Flt3 is activated by binding of its natural Flt3-ligand (Flt3-L), but Flt3 genes with internal tandem duplications (Flt3-ITD) or Asp(D)-835 point mutations encode molecules with constitutive activation. The aim of this study was to compare functional effects of exogenous Flt3-L on AML blast populations with and without genetic Flt3 abnormalities.

Design and Methods. Native AML blasts were derived from 64 consecutive patients with high blast counts in peripheral blood, and *in vitro* models were used to characterize the Flt3-L effects.

Results. The Flt3 protein levels showed a similar wide variation between AML blast populations with and without genetic Flt3 abnormalities. Flt3-L was an autocrine growth factor only for 2 patients. Flt3-ITD$^+$ AML cells had lower responsiveness to exogenous cytokines than cell populations without Flt3 abnormalities, but exogenous Flt3-L increased blast proliferation both for patients without Flt3 abnormalities and patients with Flt3-ITD as well as D835 mutations. This enhancement was observed even in the presence of other exogenous cytokines and included clonogenic AML progenitors. Flt3-L inhibited proliferation only for 1 patient, but had divergent effects on AML blast cytokine release. Flt3-L affected AML blast differentiation (inhibition of erythroid colonies, increased neutrophil granulation) only in a minority of patients, whereas it had an anti-apoptotic effect for a larger subset of patients.

Interpretation and Conclusions. Intracellular signaling initiated by Flt3 ligation modulates the functional phenotype for native human AML blasts both with and without genetic Flt3 abnormalities.

Key words: acute myelogenous leukemia, Flt3 internal tandem duplications, Flt3-D835 mutations, Flt3 ligand, cytokines, *in vitro* effects.

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Acute myelogenous leukemia (AML) is characterized by clonal proliferation of immature myeloid cells and has an overall disease-free survival after intensive chemotherapy of less than 50%.[1-4] The most important cause of death is AML relapse,[1-4] and the two most important predictors of relapse have been cytogenetic abnormalities and response to induction therapy.[2] However, recent studies have demonstrated that abnormalities of the Flt3 gene (a membrane-anchored receptor tyrosine kinase) are also associated with an increased relapse risk.[3-12] At diagnosis nearly 30% of AML patients have genetic Flt3 abnormalities,[10-12] and new abnormalities can develop later in leukemia relapse.[13,14] The adverse prognostic impact of Flt3 internal tandem duplications (Flt3-ITD) is now well established,[3,4] and recent evidence suggests that Asp(D)-835 point mutations have a similar prognostic effect.[12]

The Flt3 gene encodes for a tyrosine kinase with an extracellular ligand-binding part and an intracellular catalytic unit.[6-10] Ligation of the Flt3 molecule induces activation of the tyrosine kinase through ligand-induced receptor oligomerization and autophosphorylation with subsequent phosphorylation of cytoplasmic substrates.[5-9] However, genetic Flt3 abnormalities result in the expression of a tyrosine kinase with constitutive activity in the AML blasts.[8,9,15,16] Previous experimental evidence suggests that the constitutive activation is implicated in leukemogenesis, and recent clinical studies have demonstrated that constitutive kinase activation is also important for chemosensitivity in AML.[3,4,12]

The clinical and experimental studies discussed above suggest that intracellular signaling events initiated by Flt3 activation are important for the regulation of functional characteristics of AML blasts.[3,4,8-10] However, it is not known whether the functional effects of Flt3-ligation by the natural Flt3-L differ between AML blasts without genetic Flt3 abnormalities and leukemia cells that express abnormal Flt3 molecules with constitutive activity. The aim of the present study was, therefore, to characterize the functional effects of natural Flt3 ligation in detail in a large group of consecutive patients, and to compare these effects in native AML blasts with and without genetic Flt3 abnormalities (Flt3-ITD or D835 mutations).

Design and Methods

Patients

The study was approved by the local Ethics Committee and samples were collected after informed consent had

Table 1. Clinical and biological characteristics of AML patients.

					Membrane molecule expression[1]							
Pat.	*Sex*	*Age*	*Previous malignant or pre malignant disease*	*FAB classification*	*CD13*	*CD14*	*CD15*	*CD33*	*CD34*	*Cytogenetic analysis*	*FLT3-abnormality*[2]	*WBC counts*[3]
1.	M	44		AML-M5	+	–	+	+	+	inv(16)	PM	351
2.	F	36	Neurofibromatosis, malignant Schwannoma	AML-M5	+	–	+	+	–	t(9;11)	–	37.6
3.	M	49		AML-M4	–	–	+	+	–	nt3	ITD	78
4.	M	69		AML-M2	+	–	nt	+	–	inv(16)	–	89
5.	F	87		AML-M1	–	–	nt	+	–	nt	–	51.2
6.	M	83		AML-M1	–	–	–	+	+	nt	–	80
7.	M	72		AML-M4	+	+	+	+	–	+11	ITD	290
8.	M	49		AML-M5	+	+	nt	+	–	Normal	ITD	63.5
9.	F	58		AML-M2	+	–	+	+	–	Normal	ITD, wt^-	40.7
10.	F	56		AML-M2	+	–	–	+	–	+21	–	69.2
11.	F	38		AML-M5	+	+	+	+	+	Normal	nt	182
12.	F	55		AML-M0	+	–	–	–	+	Normal	ITD	43.6
13.	M	51		AML-M4	+	+	+	+	–	Normal	–	31.4
14.	F	49		AML-M1	–	–	–	+	+	+21	–	121
15.	M	65		AML-M1	+	–	–	+	–	Normal	ITD, wt^-	166
16.	M	64		AML-M2	+	–	+	+	+	nt	–	23.5
17.	F	63		AML-M5	–	+	+	+	–	t(2;3), (q37;q21), (q13;q21;q21) der (11q), 19q+	–	57.8
18.	F	36		AML-M5	+	+	+	+	–	Normal	nt	88.6
19.	F	82		AML-M2	+	–	–	+	+	nt	ITD	49
20.	F	63		AML-M4	–	–	–	+	–	nt	nt	126

Patients were regarded as positive when more than 20% of blast cells stained positive judged by flow cytometric analysis. All AML populations were negative for T-lymphocyte (CD2, CD3) and B-lymphocyte (CD19, CD20) markers; Flt3- abnormalities were internal tandem duplications (ITD), Asp-D835 point mutations (PM) and loss of wild type (wt^-), nt, not tested; white blood cell (WBC) counts in peripheral blood are expressed as $\times 10^9$/L (normal range 3.5-10.5$\times 10^9$/L).The WBC included at least 80% leukemia blasts.

been provided. During the period 1991-2001 AML blasts were taken from 64 consecutive patients with high peripheral blood blast counts. The patients were classified as having AML-M0/M1 (21 patients), AML-M2 (20 patients) and AML-M4/M5 (23 patients). Forty-six patients had newly diagnosed *de novo* AML, 6 patients had AML relapse, and 12 patients had AML secondary to chemotherapy (4 patients), chronic myeloproliferative disorders (2 patients) or primary myelodysplastic syndromes (6 patients). Leukemic cells from the last 20 patients were used in most experiments, and the characteristics of these patients are presented in Table 1.

Cytogenetic analyses were performed for the last 48 patients included in our study; of these, 28

patients had a normal karyotype, 3 patients had a favorable karyotype (all inv(16)) and 5 had an unfavorable karyotype according to the definitions used by Wheatley *et al.*[2] and Kottaridis *et al.*[3] A total of 98 patients with AML were admitted to our institution during the same period. The distribution of karyotypes in the patients selected for our present study did not differ significantly from that in the whole series of patients seen during the same period. The relatively low frequency of patients (3/48) with favorable karyotypes is different from that reported in other studies[2,3] and was also observed for the whole series of patients from the same period (6/98).

Preparation of AML blasts

Native AML blasts. Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; NyCoMed, Oslo, Norway; specific density 1.077) from the peripheral blood of patients with a high percentage of AML blasts among blood leukocytes (Table 1). Cells were stored frozen in liquid nitrogen.[23] The percentage of blasts among leukemic PBMC exceeded 95% for all patients,[24-26] the contaminating cells being small lymphocytes.

Enriched AML blasts. Immunomagnetic beads coated with anti-CD2 and anti-CD19 specific monoclonal antibodies (Dynabeads; Dynal, Oslo, Norway) were used for depletion of $CD2^+$ and $CD19^+$ cells, respectively.[24] Depletion was performed in two separate steps before adherent cells were removed, and the enriched populations contained <1% of $CD2^+$ T-cells and $CD19^+$ B-cells.[24]

Analysis of Flt3 abnormalities in AML blasts

Our method for analysis of Flt3-ITD has recently been described in detail.[14] Analysis of D835 point mutations (PM) was performed using the restriction fragment gene length polymorphism at codon 835/836 as described previously.[27] Briefly, after amplification of a 111 bp fragment from exon 20 using genomic DNA with primer 20F-5'-CCGCCAGGAACGTGCTTG-3' and 20R-5'-GCCTCACATTGCCCCTGA-3', polymerase chain reaction (PCR) products were digested by EcoRV and the fluorescence (6-FAM, 6-carboxylfluorescein)-labeled product analyzed.[14] Undigested products served as template for a new polymerase chain reaction with the same primer combination, and the products were thereafter cleaned using ExoSAP-IT before being directly sequenced using an ABI BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

Analysis of Flt3 expression

Cell suspensions were lysed at 4°C in 60-100 μL of lysis buffer (10 mM trishydroxymethyl-aminomethane (Tris) with pH 7.5, 400 mM NaCl, 10% glycerol, 0.5% detergent Nonidet P-40 (Amersham Biosciences, Uppsala, Sweden), 5 mM NaF, 0.5 mM Na-orthovanadate, 1 mM dithiotreitol (DTT), and protease inhibitor cocktail Complete (Roche, Basel, Switzerland). Samples were kept at 4°C, homogenized and centrifuged (14,000g, 15 minutes). 3X sodium dodecyl sulphate (SDS) loading buffer (0.5 M Tris pH 6.8, 2 M β-mercaptoethanol, 12% SDS, 30% glycerol, bromphenol blue) was added to supernatant aliquots containing 40 μg of protein, and thereafter boiled for 7 minutes before separation in SDS-polyacrylamide gel electrophoresis (PAGE) minigels with 7.5% polyacrylamide. After electroblotting to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences) and blocking for 1 h in phosphate-buffered saline with 0.5% Tween (PBS-T), the filters were incubated with primary anti-Flt3 antibody (the rabbit polyclonal S18 and C20 antibodies diluted 1:250 in PBS-T; Santa Cruz, CA, USA) for 1 h (room temperature) or overnight (4°C) before washing for 1 hour in PBS-T. Both antibodies recognized the p160 and p130 isoforms of Flt3. The C-20 antibody reacts with a C-terminal epitope whereas S-18 reacts with the kinase insert region. The PVDF membranes were thereafter washed (1 hour in PBS-T), incubated for 1 hour with a secondary anti-rabbit antibody conjugated to alkaline phosphatase (the antibody dissolved in PBS-T), washed (1 hour in PBS-T), and finally incubated with CDP-Star Chemiluminisence Substrate (Applied Biosystems, Foster City, CA, USA). The membranes were then exposed to Kodak X-ray films which were scanned for densiometric analysis of the 130 plus 160 kDa bands (Microtek Scanmaker 5700, NIH Image ver. 1.60 for Apple Macintosh). The intensity for each AML sample was normalized to the lower 70 kDa anti-Flt3-reactive band in Jurkat control extracts which were included in each gel (equal intensity defined as 1.0). Equal protein loading was confirmed by staining the minigels with Coomassie blue. Actin could not be used as the loading control because of differences in the molecular weight of the immunoreactive bands between patients.

Reagents for tissue culture

Cytokines. Recombinant human Flt3-L (Peprotech; Rocky Hill, NJ, USA) was used at a concentration of 20 (only ^{3}H-thymidine incorporation) or 50 ng/mL; this was based on previous studies of *in vitro* cultured AML blasts showing that Flt3-L effects reach a plateau at concentrations ≥10 ng/mL.[8,18,21] Other recombinant human cytokines were used at the following concentrations: interleukin 1β (IL1β, Peprotech) 50 ng/mL; IL3 (Peprotech) 20 ng/mL, stem cell factor (SCF; Peprotech) 20 ng/mL, thrombopoietin (TPO; Peprotech) 50 ng/mL, vascular endothelial growth factor (VEGF;

Peprotech) 50 ng/mL, macrophage colony-stimulating factor (M-CSF, Peprotech) 50 ng/mL, granulocyte-macrophage colony-stimulating factor (GM-CSF; Sandoz, Basel, Switzerland) 100 ng/mL, G-CSF (Roche) 100 ng/mL.

Culture media. Unless otherwise stated, the culture medium was RPMI 1640 with HEPES and glutamine (BioWhitacker; Walkersville, MA, USA) and supplemented with 10% inactivated fetal calf serum (FCS; BioWhitacker).[26] The serum-free media X-vivo 10®, X-vivo 15® (BioWhitacker) and StemSpan SFEM™ (referred to as StemSpan; Stem Cell Technologies, Vancouver, BC, Canada) were used in certain experiments.[26] All the media contained 100 μg/mL of gentamicin.

Antibodies. The monoclonal Flt3-L specific neutralizing antibody (clone 40416.111; R&D Systems, Abingdon, UK) was always tested in parallel with an isotypic control antibody; 0.02-0.06 μg/mL of this anti-Flt3-L antibody will neutralize 50% of the bioactivity of 5 ng/mL of recombinant human Flt3-L (manufacturer's information).

Assays for AML blast proliferation

Suspension cultures. As described previously,[24-26] 5×10^4 cells/well were cultured in 150 μL medium in flat-bottomed microtiter plates (Costar 3796; Cambridge, MA, USA). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2. After six days ^{3}H-thymidine (37 kBq/well; TRA 310, Amersham International, Amersham, UK) was added in 20 μL of 0.9% NaCl solution and nuclear radioactivity assayed 18 hours later by liquid scintillation counting.

Colony formation assays. AML blasts were cultured in different methylcellulose-based media: (i) medium alone (MethoCult H4230; Stem Cell Technologies, referred to as spontaneous colony formation) or the same medium supplemented with GM-CSF (GM-CSF-dependent colony formation); (ii) medium with erythropoietin plus phytohemagglutinin-leukocyte conditioned medium (MethoCult H4433; Stem Cells Technologies). Cells were cultured in 24 well tissue culture plates (Costar 3524) with 10^5 cells in 0.5 mL medium per well. Cultures were incubated for 14 days before the number of colonies containing at least 20 cells was determined by light microscopy (duplicate analysis). The colonies were classified as erythroid (red color in the whole or a part of the colony) and non-erythroid.

Cytokine analysis

Analysis of AML cell cytokine secretion. As described previously,[25] 1×10^6 AML blasts/mL were cultured in 24-well tissue culture plates (Costar 3524; 2 mL medium/well) for 48 hours before supernatants were harvested. ELISA analyses were used to determine levels of IL1β, IL6, tumor necrosis factor (TNF) α (Pelikine compact ELISA kits; Central Laboratory of the Netherlands' Red Cross Blood Transfusion Services, Amsterdam, The Netherlands), Flt3-L, G-CSF and GM-CSF (Quantikine ELISA kits; R&D Systems) in the supernatants. The minimal detectable levels were IL1β 0.8 pg/mL, IL6 0.8 pg/mL, TNFα 1.0 pg/mL, Flt3-L 7 pg/mL, GM-CSF 3 pg/mL and G-CSF 8 pg/mL.

Cytokine-specific RNA levels. AML blasts (2×10^6 cells in 2 mL FCS-containing medium per well; Costar 3524 culture plates) were cultured for 48 hours before cells were harvested and washed in phosphate-buffered saline. The cell pellets were stored frozen at -70°C until total RNA was isolated.[29] For quantification of IL1β- and IL6-specific RNA the samples and calibrators were hybridized in microwells with gene-specific biotin-labeled capture oligonucleotide probes and digoxigenin-labeled detection probes, and cytokine-specific RNA levels then determined in a calorimetric microplate assay (Quantikine RNA assay, R&D Systems). The results are expressed as concentrations of IL1β- and IL6-specific RNA when testing total RNA at the concentration of 2.5 μg/mL.

Studies of apoptotic cell death

Estimation of the number of apoptotic cells. AML blasts were incubated for 24 and 48 hours before cell death was analyzed as described in detail previously.[24,25,30-32] Firstly, AML blasts were stained with DNA-specific bisbenzimide H33258 (Hoechst; Basel, Switzerland) or daunorubicin (Pharmacia), and the percentage of cells showing chromatin distribution consistent with apoptosis was determined by fluorescence microscopy.[25,30] Secondly, detection of phosphoserine exposure on the cell surface was used as a marker for apoptosis; flow cytometric analysis was then performed on FITC-annexin V stained cells as described previously.[31] Thirdly, JC-1 staining (Molecular Probes) was used to determine the mitochondrial status, and the number of cells with depolarized mitochondria consistent with apoptosis was determined by flow cytometric analysis.[31,32]

Caspase-3 activity in AML cells. Caspase-3 activity was measured in cell extracts using a specific caspase-3 cellular activity assay kit (Calbiochem; La Jolla, CA, USA). Briefly, 1×10^6/mL AML blasts (2 mL of medium per well, 24-well Costar 3524 culture plates) were cultured for 48 hours before cells were harvested, washed twice, and cell concentration adjusted to 1×10^6 cells/mL in lysis buffer. These cell extracts were stored at -70°C until enzymatic activity was assayed according to the manufacturer's instructions. The results are presented as pmol/min for 80 μL of sample volume.

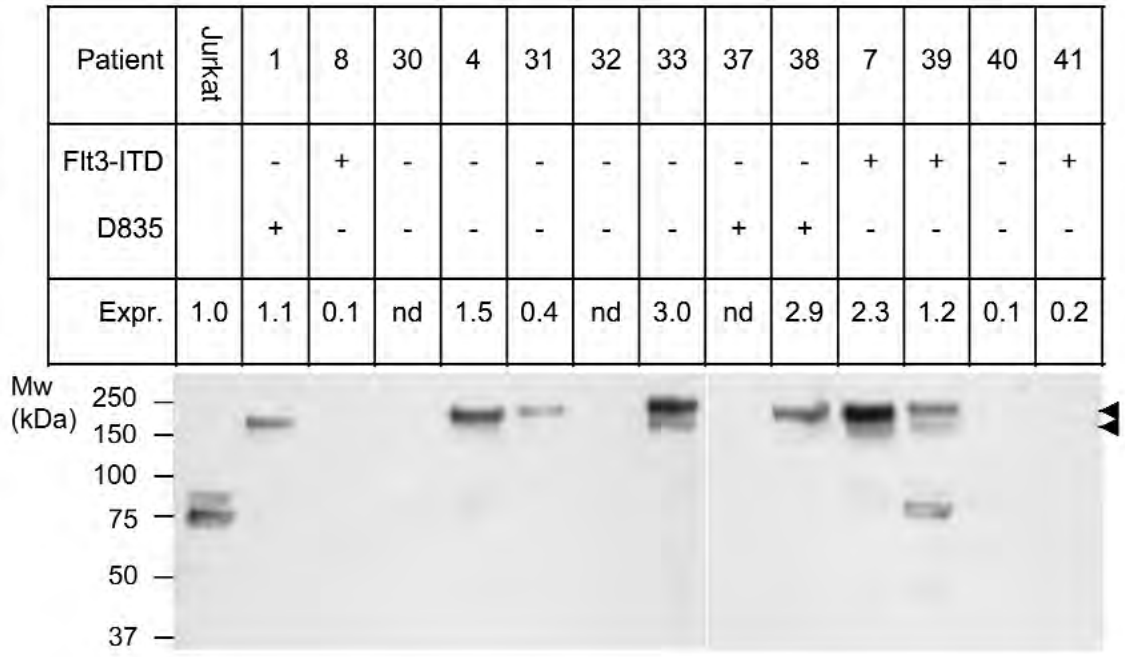

Figure 1. Protein expression of Flt3 by Jurkat cells and native AML blasts derived from 12 patients. An antibody reactive with the C-terminal domain (C-20 antibody) was used as described in Design and methods. The molecular weight (Mw) in kDa is indicated on the left side, and the arrows on the right side indicate that the antibody bound two bands corresponding to the 130 and 160 kDa isoforms. The patients' number is given at the top of the figure. For each individual patient it is also indicated whether Flt3-ITD was detected, and detection of D835 mutations is indicated by +. The Flt3 protein level is presented as the relative expression (Expr) compared with the 70 kDa band of the Jurkat cell (nd, not detectable). The figure illustrates the wide variation in the expression of both the 130 and 160 kDa bands.

Presentation of the data

^{3}H-thymidine incorporation was assayed in triplicate and the mean counts per minute (cpm) used for all calculations. The *incremental response* was defined as the cpm for cultures with AML blasts minus cpm for negative controls, and significant blast proliferation was defined as an incremental response exceeding 1,000 cpm. A *significant alteration* of proliferation was defined as a difference in incremental responses (i) exceeding 2000 cpm, and (ii) the difference in cpm being >20% of the control response. For cytokine combinations an *additive enhancing* or *inhibitory* effect was defined as a proliferative response exceeding the highest/lowest of the two single responses by at least 2000 cpm and 20%; smaller differences are referred to as intermediate. A significant *alteration of AML blast colony formation* was defined as a difference corresponding to >20% of the control response and with an absolute value >10 per 10^5 seeded cells. Cytokine concentrations were transformed to logarithmic values that were used for statistical comparisons. The Sign test, χ^2 test and Wilcoxon's test for paired samples were used for statistical analysis, and differences were regarded as significant when $p<0.05$.

Results

Flt3 protein expression in native AML blasts

The protein level of Flt3 in native AML blasts was examined for 42 patients. Two molecules corresponding to the 130 and 160 kDa isoforms of Flt3 were detected by both antibodies. The protein level of Flt3 showed a wide variation between patients, and the relative expression ranged from not detectable to 3.0. The relative levels of Flt3 did not differ significantly between Flt3-ITD$^+$ (median 0.31, range 0.01-2.40) and ITD$^-$ (median 0.38, range <0.01-3.00) AML cell populations. A wide variation was also observed for the 3 ITD$^-$PM$^+$ patients (range <0.01-1.5). This is illustrated by the results presented in Figure 1.

The proliferative capacity of AML blast populations with and without Flt3 abnormalities

AML blasts derived from 55 patients were available for analysis of Flt3 abnormalities. Flt3-ITD was detected for 21 patients (38%). A D835 point mutation was detected for 7 patients (13%), and 3 of these patients had both ITD and point mutations.

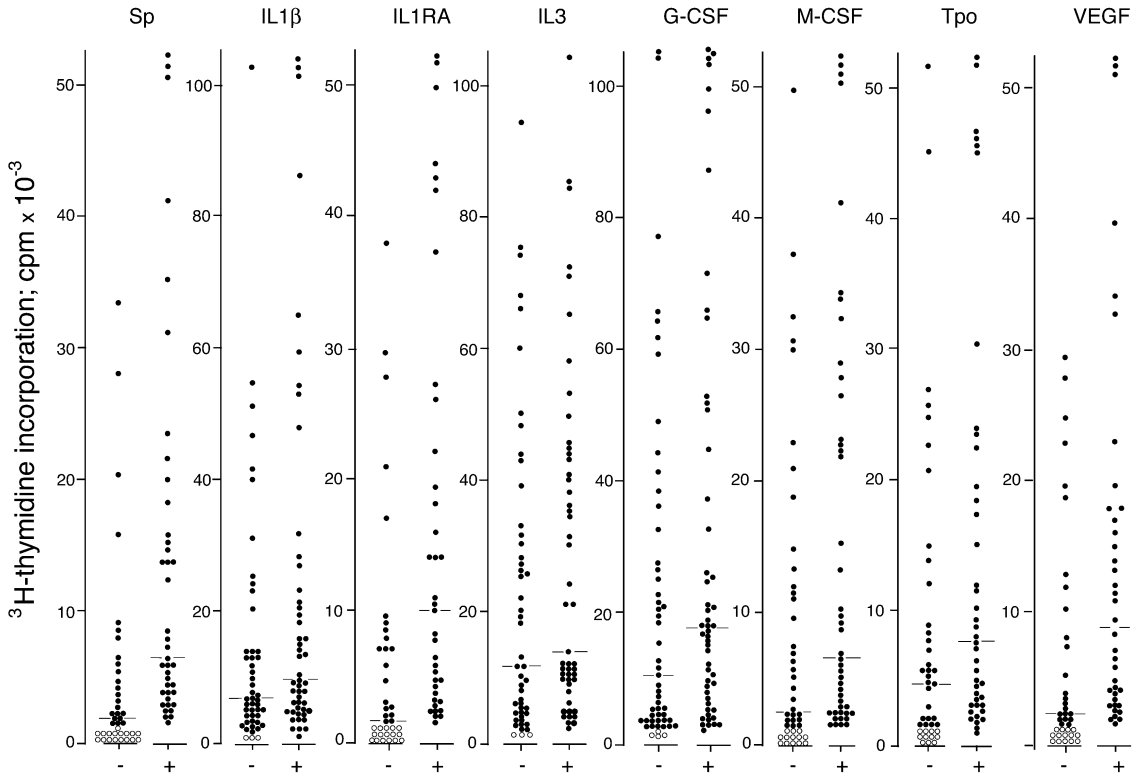

Figure 2. The effect of Flt3-L on AML blast proliferation. Leukemia blasts were derived from 64 consecutive patients, but the figure presents the results only for those patients showing detectable ^{3}H-thymidine incorporation for cultures without (⁻) or with (⁺) Flt3-L 20 ng/mL. AML blasts were cultured either in medium alone (Sp, 40 out of 64 patients showing detectable proliferation) or in the presence of IL1β (49/64), IL1RA (37/64), IL3 (49/64), G-CSF (52/64), M-CSF (44/64), TPO (41/64) or VEGF (38/64). All these exogenous cytokines were tested at 50 ng/mL except G-CSF that was tested at 100 ng/mL. The results for each patient are presented as the mean cpm of triplicate determinations, and undetectable proliferation is indicated in the figure (o). The median ^{3}H-thymidine incorporation for all these patients is also indicated in the figure (—).

The presence of Flt3-ITD showed no association with FAB classification, the expression of the stem cell marker CD34 or the ability of autocrine proliferation (*data not shown*). We also investigated the proliferative capacity in the presence of exogenous cytokines (IL1β, IL3, G-CSF, M-CSF, GM-CSF, SCF, Flt3-L, TPO, VEGF). This wide range of cytokines was used because the growth factor responsiveness of AML populations is heterogeneous. A total of 495 patient/cytokine combinations were thus examined. The frequency of combinations with undetectable ^{3}H-thymidine incorporation (<1000 cpm) was significantly higher for Flt3-ITD⁺ patients (n=21, 90 out of 189 combinations) than for Flt3⁻ITD⁻ patients (n=34, 89 out of 306 combinations, χ^2 test, $p<0.001$). This difference was also statistically significant when Flt3-PM⁺ patients were excluded from the control group. Undetectable ^{3}H-thymidine incorporation in the presence of all nine cytokines was most common among patients with Flt3 abnormalities (Flt3-ITD⁺ or PM⁺ 6/21, no Flt3 abnormalities 4/34), but this difference did not reach statistical significance.

Effects of exogenous Flt3-L on proliferation of AML blasts in suspension cultures

The proliferation of native AML blasts derived from 64 consecutive patients was assayed when cells were cultured with and without exogenous Flt3-L 20 ng/mL in FCS-containing medium alone and medium with various exogenous cytokines. AML blasts from 10 patients did not proliferate *in vitro* either in medium alone or in the presence of any exogenous cytokine. The overall results for the other 54 patients with detectable ^{3}H-thymidine incorporation (corresponding to >1000 cpm) are presented in Figure 2 and summarized in the upper part of Table 2. When the statistical analysis only included those patients with detectable proliferation (>1000 cpm), (i) Flt3-L increased AML blast proliferation significantly both for cells cultured in medium alone and cells cultured with exogenous IL1β, IL1RA, IL3, G-CSF, M-CSF, TPO, and VEGF (Sign test, $p<0.002$ for each), and (ii) the enhancement reached a significant level (for definitions see Design and methods, presentation of the data) for

Table 2. Effects of Flt3-L on *in vitro* proliferation of native AML blasts; studies of ^{3}H-thymidine incorporation of AML blasts derived from 64 patients.

Comparison (patient number)	*Effect*	*Exogenous cytokine added alone and together with Flt3-L[1] (number of patients)*							
		None	*IL1*	*IL1RA*	*IL3*	*G-CSF*	*M-CSF*	*TPO*	*VEGF*
Cultures with exogenous Flt3-L compared with corresponding controls (n=64)	Increase[2]	34	26	29	29	31	23	26	28
	Intermediate[2]	5	21	7	17	15	20	14	8
	Decrease[2]	1	1	1	3	6	1	1	2
	No detectable proliferation[2]	24	16	27	15	12	20	23	26
Cultures with exogenous Flt3-L compared with corresponding controls (n=64)	Additive growth-enhancing effects[3]	–	15	0	27	28	16	14	2
Flt3-ITD$^+$ AML blasts (n=21)[4]	Flt3-L induced increase of proliferation[2]	9	8	7	9	13	7	7	6
Flt3-PM$^+$ AML blasts (n=4)[4]	Flt3-L induced increase of proliferation	2	2	3	2	1	1	1	2
Flt3-ITD$^-$ AML blasts (n=30)[4]	Flt3-L induced increase of proliferation	19	13	16	12	15	12	14	15

The results for 64 patients are presented.[1] For each cytokine the effects were classified (see below) and the number of patients showing enhanced/indifferent/decreased/no significant proliferation in the presence of exogenous Flt3-L is given for each cytokine. The effect of Flt3-L was investigated for AML blasts cultured in medium alone or medium with exogenous cytokines (IL1β, IL1RA, IL3, G-CSF, TPO, M-CSF, VEGF). The upper part of the table describes the effect of exogenous Flt3-L in the presence of other mediators, the middle part states the number of patients with an additive effect of Flt3-L and other exogenous cytokines (for detailed definitions see Design and Methods, presentation of the data), and the lower part compares the numbers of patients with Flt3-L-induced growth enhancement for Flt3-ITD$^+$ and -ITD- AML blast populations.[2] A significant alteration was defined as a difference being (i) >20% of the corresponding Flt3-L-free control and (ii) with an absolute value >2000 cpm. Smaller differences were classified as intermediate. No detectable proliferation was defined as ^{3}H-thymidine incorporation <1000 cpm both for cultures with and without Flt3-L.[3] An additive effect of Flt3-L and another cytokine was defined as a proliferative response exceeding the highest of the two responses by at least 20% and this difference being >2000 cpm.[4] Nine patients were not available for Flt3-ITD testing. The group of Flt3-ITD$^+$ patients includes 3 patients with additional Flt3-PM, whereas patients with Flt3-PM alone are presented as a separate group.

a large group of patients both when cells were cultured in medium alone and when they were cultured with medium and exogenous cytokines (Table 2, upper part). These differences were also statistically significant when all patients were included in the analysis (*data not shown*). Flt3-L and the other cytokines had additive effects only for a subset of the patients (Table 2, middle part). When comparing the overall results for the 54 patients with detectable proliferation, Flt3-L had significant effects on blast proliferation in medium alone or in the presence of at least one exogenous cytokine for 50 of these patients. However, the Flt3-L effect showed no correlation with the Flt3 protein levels (*data not shown*), and the growth-enhancing effect was detected for blast populations both with and without Flt3 abnormalities (Table 2, lower part).

AML blast expression of wild-type Flt3 could not be detected for 3 ITD$^+$ patients. However, exogenous Flt3$^-$L could modulate AML blast proliferation even for these patients (Table 3).

The effects on spontaneous and cytokine-dependent blast proliferation were also compared for Flt3-L, GM-CSF and SCF (Figure 3). All three cytokines caused strong enhancement of the proliferation for a majority of patients both when cells were cultured in medium alone and when the cells were cultured with medium in the presence of exogenous cytokines (Figure 3). Flt3-L (Table 2), GM-CSF and SCF (*data not shown*) had additive growth-enhancing effects with other cytokines only for a subset of patients.

Enriched AML blasts were prepared for 5 patients (Table 1, patients #7, 8, 11, 14 and 20), and Flt3-L increased the proliferation of enriched cells for all these patients (*data not shown*).

Flt3-L as an autocrine growth factor for native AML blasts

AML blasts derived from 64 patients were cultured for 48 hours before concentrations of Flt3-L were determined in the supernatants. Flt3-L did not reach detectable levels (<7 pg/mL) for any patient. Leukemia cells derived from the 9 patients with the highest spontaneous *in vitro* proliferation were also cultured with Flt3-L specific monoclonal antibody and isotypic control antibodies. Anti-Flt3-L caused a dose-dependent inhibition of spontaneous blast proliferation only for patient 8 (Flt3-ITD$^+$PM$^-$) and 10 (Flt3-ITD$^-$PM$^-$), but anti-Flt3-L did not have an antiproliferative effect for any patient in the presence of exogenous GM-CSF (Figure 4).

Table 3. Effects of exogenous Flt3-L on *in vitro* proliferation of native AML blasts: studies of Flt3-ITD+ leukemia cells with undetectable expression of wildtype Flt3.

Patient and culture characteristics	*Flt3-L 20 ng/mL*	*Patient 9*	*Patient 15*	*Patient 24*
Flt3-ITD (base pairs)		24	63/33	48
Flt3-PM (D835)		–	–	–
Exogenous mediator				
None	–	1084±172	147±29	299±27
	+	**5891±998**	696±98	497±98
IL1	–	3456±627	568±108	14.836±1982
	+	**7090±882**	939±111	17.005±2035
IL1RA	–	478±87	183±23	485±82
	+	**2769±524**	724±74	612±54
IL3	–	68.911+3760	542±225	8653±1230
	+	80.511+4072	939±339	**11.783±1074**
G-CSF	–	2156±524	524±92	21.284±2310
	+	**10.330±1099**	1706±233	21.058±1962
M-CSF	–	564±38	510±44	539±27
	+	**3166±422**	833±92	591±83
TPO	–	378±88	228±76	4836±623
	+	**2962±499**	876±73	4471±826
VEGF	–	259±78	127±29	312±27
	+	**2654±881**	594±127	483±99

AML blasts were cultured in suspension cultures with and without Flt3-L 20 ng/mL. The Flt3-L effects were assayed for cells cultured in medium alone and medium supplemented with various exogenous cytokines. ^{3}H-thymidine incorporation was assayed after 7 days of culture, and the results are presented as the mean+standard deviation of triplicate determinations. Results in bold represent significant alterations induced by Flt3-L (the difference corresponding to >20% of the controls and an absolute value >2,000 cpm).

Table 4. The effect of Flt3-L on clonogenic AML cells; effects of pre-incubation with Flt3-L before analysis of colony formation.

			Colony-formation with erythropoietin plus conditioned medium (number of colonies per 10^5 seeded cells)	
Patient	*FLT3-abnormality*	*Flt3-L 50 ng/mL*	*Non Erythroid*	*Erythroid*
1.	PM	–	16.5±0.7	0
		+	17.5±0.7	0
2.	–	+	117.0±26.9	150.0±6.3
			115.0±9.9	**170.0±14.0**
3.	ITD	–	85.0±15.4	0
		+	**125.0±4.2**	2.0±1.4
4.	–	–	170.0±11.3	96.0±25.4
		+	**144.0±11.3**	**23.0±7.0**
5.	–	–	194.0±31.1	30.0±12.5
		+	**265.0±29.6**	**1.0±1.4**
6.	–	-	15.0+712.5	0
		+	**123.0+12.6**	0
7.	ITD	–	41.5±3.5	0
		+	**91.0±14.7**	0
8.	ITD	–	118.0±14.1	71.5±18.3
		+	**168.0±24**	**0.5±0.7**
9.	ITD, wt–	–	72.0±25.5	23.0±9.1
		+	**126.0±1.4**	**1.5±0.7**
10.	–	–	329.0±43.8	0
		+	**371.0±49.5**	0
12.	ITD	–	39.0±5.6	4.0±2.8
		+	**64.0±21.0**	1±1.4
13.	–	–	24.5±2.1	23.5±10.5
		+	28.0±0	23.0±13.3
14.	–	–	57.7±6.3	0
		+	**45.0±2.8**	0
15.	ITD, wt-	–	82.0±24.8	3.0±2.8
		+	**154.0±4.2**	0
16.	–	–	176.0±34.3	0
		+	**127.5±33.6**	0
17.	–	–	4.5±3.5	4.0±5.6
		+	4.0±5.6	**44.5±9.1**
18.	nt	–	50.0±4.2	0
		+	**90.0±7.0**	0

Cells were pre-cultured in suspension cultures with and without Flt3-L 50 ng/mL for 7 days before the number of colonies was determined in the erythropoietin-conditioned medium assay. Values for patients with significant effects of Flt3-L are marked in bold: this was defined as differences >10 and exceeding the Flt3-L-negative control by 10%. The results are presented as the mean+SD of duplicate determinations.

Effects of exogenous Flt3-L on AML blast colony-formation

AML blasts derived from 17 patients (Table 1, patients #1-10 and 12-18) were pre-incubated in serum-free StemSpan™ medium without and with Flt3-L 50 ng/mL for 7 days before the frequency of colony-forming cells was determined under Flt3-L-free conditions by using the erythropoietin+conditioned medium assay. Patients with Flt3 abnormalities showed lower frequencies of clonogenic cells than did the other patients, but this difference did not reach statistical significance (Table 4). For most patients Flt3-L either increased (10 of 17 patients) or did not alter (4/17) the frequencies of non-erythroid colonies, but a reduction was observed for 3 patients. Erythroid colonies were detected for 10 patients; pre-incubation with Flt3-L reduced this frequency for 4 patients and increased it for 2 patients. The Flt3-L effects did not differ between

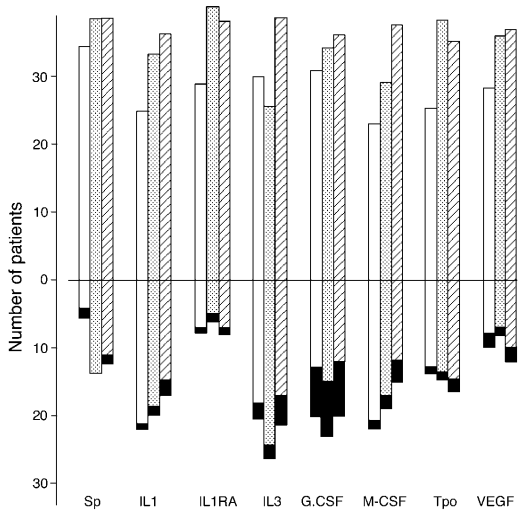

Figure 3. Effects of Flt3-L, GM-CSF and SCF on AML blast proliferation. The proliferation of native AML blasts cultured in medium alone or with Flt3-L 20 ng/mL (open columns), GM-CSF 100 ng/mL (stippled) or SCF 20 ng/mL (stripes) was first compared (Sp, i.e. spontaneous proliferation in medium alone). The effects of these three cytokines were also examined when the culture medium was supplemented with other exogenous cytokines (IL1, ILβ1RA, IL3, G-CSF, M-CSF, TPO, VEGF; see the bottom of the figure), and proliferation in Flt3-L/GM-CSF/SCF containing cultures was then compared with that in the corresponding cytokine-containing controls. An increase or decrease in blast proliferation was defined as an alteration corresponding to (i) >20% of the corresponding control and (ii) exceeding 2000 cpm; smaller differences are referred to as intermediate responses. A total of 64 patients were examined, and the figure presents the results for those patients who showed detectable proliferation (corresponding to >1000 cpm) for each of the cytokines. The figure shows the number of patients with increased (the part of the columns above the X-axis) and intermediate/decreased (the part of the column below the X-axis) proliferation in the presence of Flt3-L/GM-CSF/SCF. The number of patients with decreased responses is indicated in black at the lowest part of each column.

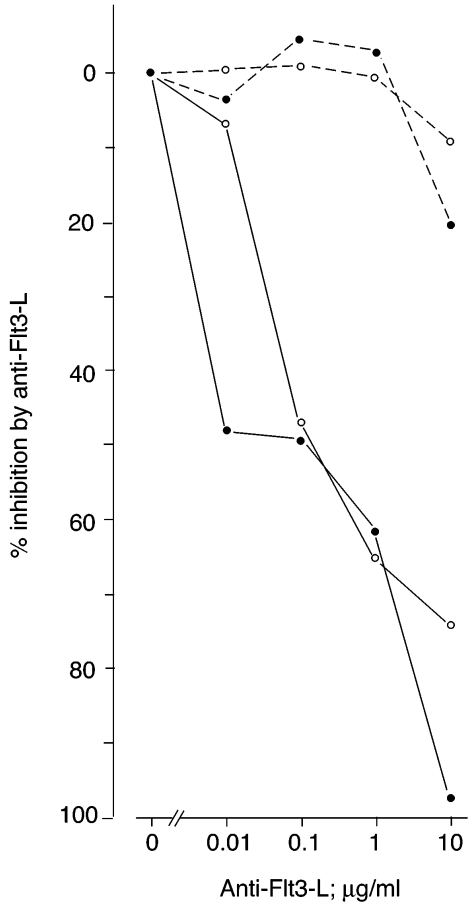

Figure 4. The effect of Flt3-L specific neutralizing antibodies on spontaneous and GM-CSF-dependent AML blast proliferation. Native AML blasts derived from two patients (Table 1; patients 8 •, 10 o) were cultured either in medium alone (____) or in the presence of GM-CSF 100 ng/mL (—). Proliferative responses were then compared for cultures with Flt3-L-specific neutralizing monoclonal antibodies and cultures containing isotypic control antibodies at the same concentration (see x-axis). The results are presented as the percent inhibition by neutralizing antibodies, i.e. the incremental cpm for cultures with Flt3-L specific antibodies, relative to the incremental cpm for corresponding isotypic control cultures. The responses in isotypic control cultures were: patient #8, spontaneous proliferation 8302+1876 cpm, GM-CSF dependent proliferation 36.813+852 cpm; patient # 10, spontaneous proliferation 17.206+1428 cpm, GM-CSF dependent proliferation 94.865+3474 cpm.

patients with and without Flt3 abnormalities and were reproduced for 5 patients (Table 1, patients 1#, 2, 6, 8, 9) when using various media for the pre-incubation (RPMI + 10% FCS, X-vivo 10®, X-vivo 15®, StemSpan™, *data not shown*). Spontaneous and GM-CSF-dependent colony formation were examined for 8 patients (Table 1, patients #7-14); the assays were then prepared with and without Flt3-L 50 ng/mL. Only non-erythroid colonies were detected. Flt3-L could increase both spontaneous (1 out of 8 patients) and GM-CSF-dependent (3/8) colony formation, and decreased colony-formation was not observed for any patients.

Effects of exogenous Flt3-L on constitutive cytokine secretion by native AML blasts

Flt3-L 50 ng/mL had divergent effects on the release of IL1β, IL6, TNFα, G-CSF and GM-CSF by native human AML blasts (Figure 5). This divergence was observed for native blasts and was

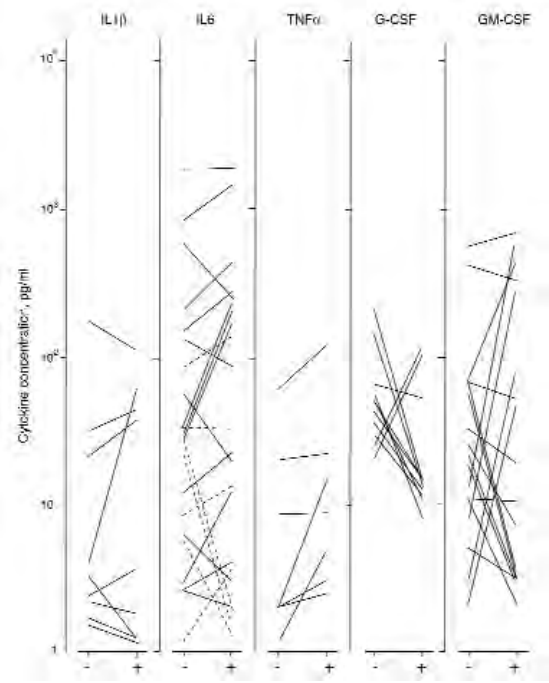

Figure 5. The effect of Flt3-L on AML blast cytokine secretion. The AML cells were cultured without and with Flt3-L 50 ng/mL, and cultures were prepared with either native (____) or enriched (----) AML blasts. The cells were cultured at 1×10^6 cells/mL for 48 hours before supernatants were harvested and cytokine levels (IL1β, IL6, TNFβ, G-CSF, GM-CSF; see top of the figure) determined by ELISA analyses. The figure compares the cytokine levels (pg/mL) for cultures without (⁻) and with (⁺) Flt3-L, and only the results for patients showing detectable cytokine levels for at least one of these cultures are presented: - IL1β: 25 patients tested, 9 patients showed detectable levels; - IL6: 15 out of 25 patients showed detectable levels; in addition the figure presents the results for enriched AML blasts derived from 7 of these patients (----); - TNFα: detectable levels were observed for 7 out of 25 patients; - G-CSF, 11 out of 19 patients showed detectable levels; - GM-CSF, 17 out of 19 patients showed detectable levels.

reproduced for enriched leukemia cells (7 patients examined), and neither the cytokine levels nor the divergent effects of exogenous Flt3-L differed between patients with and without Flt3 abnormalities. Exogenous SCF also had divergent effects on the secretion of IL1β, IL6 and TNFα (*data not shown*). Furthermore, AML blasts derived from 5 patients (Table 1, patients 1-4 and 7) were cultured with and without Flt3-L 50 ng/mL for 48 hours before IL1β- and IL6-specific RNA was quantified. IL1β- (range 15.0-39.0 amol/μg total RNA) and IL6-specific RNA (range 32.0-86 amol/μg RNA) were detected for all patients, and Flt3-L had divergent effects on these levels (*data not shown*).

Effects of exogenous Flt3-L on AML blast morphology

Native AML blasts derived from 10 consecutive patients (Table 1, patients 1-10) were cultured with and without Flt3-L 50 ng/mL for 2 and 7 days before examination of cell morphology (May-Grünwald-Giemsa staining). After 2 days neutrophil differentiation was observed in ≤ 10% of the cells for all patients, and these percentages were not significantly altered (i.e. ≤ 5%) by Flt3-L. In contrast, after 7 days of culture with Flt3-L an increased percentage of cells with neutrophil granulation was observed for patients 1 (ITD^-PM^+, 17 versus 5%), 2 (ITD^-PM^-, 28 versus 1%), 4 (ITD^-PM^-, 12 versus 1%) and 7 (ITD^+PM^-, 8 versus 2%). However, neutrophil differentiation further than the promyelocyte stage and erythroid or monocytoid differentiation was not observed for any patient.

Effects of Flt3-L on regulation of apoptosis in native AML cells

The frequency of apoptotic AML blasts (H33258 staining) after 24 and 48 hours of culture was first examined for 11 patients (Table 1, patients 1 and 10-19), and this frequency exceeded 30% only for patient 17 (ITD^- PM^-, 54% after 48 hours). Flt3-L reduced apoptosis significantly (i.e. ≥5% difference) for patients 1 (ITD^- PM^+), 10 (ITD^- PM^-), 13 (ITD^- PM^-), 17 (ITD^- PM^-) and 18 (ITD not tested, PM^-). Furthermore, the effects of Flt3-L, GM-CSF and SCF on apoptosis were compared for an additional group of 12 consecutive patients, 4 of whom were $Flt3^-ITD^+$ and 1 of whom was $Flt3^-PM^+$ (chromatin staining, JC-1 staining, annexin-V labeling). For $Flt3^-$L the morphologic examination demonstrated reduced apoptosis (≥5% alteration) for 6 of the 12 patients, whereas increased apoptosis was observed for 2 patients. A similar divergence was observed both after 24 and 48 hours of culture and with all three methods (*data not shown*). The anti-apoptotic effect was observed for AML blast populations both with and without Flt3 abnormalities (including the $Flt3^-PM^+$ patients). SCF and GM-CSF also had divergent effects on apoptosis (*data not shown*).

Caspase-3 activity in AML blasts was examined after 48 hours of *in vitro* culture with and without $Flt3^-$ L 50 ng/mL for patients 1 (ITD^- PM^+), 10 (ITD^- PM^-) and 19 (ITD^+ PM^-). These patients were selected because Flt3-L increased the blast proliferation both in cultures with medium alone and in cultures in the presence of all the exogenous cytokines. In contrast, Flt3-L had divergent effects on caspase-3 activity and caused either minimally altered (patient 10: without Flt3-L 34.8 pmol/min, with Flt3-L 42.9 pmol/min), decreased (patient 1: 107 versus <13.4 pmol/min) or increased activity (patient 19: 6.7 versus 68 pmol/min). Caspase-inhibitory activity was not detected for any patient.

Discussion

In our present study we included consecutive patients with high peripheral blood blast counts, and thus highly enriched populations of native AML blasts could be prepared by density gradient separation from peripheral blood samples. This simple technique has a minimal risk of inducing functional alterations in the blasts,[35,36] but a high degree of *leukemization* may reflect biologic differences[2,37] and thus our results are only representative of this particular subset of patients. The cytogenetic studies demonstrated a normal karyotype in nearly 60% of our patients, whereas favorable and unfavorable karyotypes were detected only for small subsets of patients (6% and 11% respectively). This distribution is not much different from that found in other studies, except for the low frequency of favorable karyotypes, which was also observed when we analyzed all patients admitted to our institution during the same study period.[2,3] However, the relatively high frequencies of Flt3-ITD (38 versus 27%)[3] and D-835 mutations (13 versus 7%),[28] in our present study possibly reflect a selected patient population. The relatively low number of patients examined may then explain why the frequencies of abnormal karyotypes did not differ from that found in our overall population and in other studies.[2,3]

Previous studies have reported a wide variation in the percentage of patients with Flt3-L-responsive AML blasts (range 62-88%).[22] In contrast to these studies we examined the effects of Flt3-L in a large group of consecutive patients. We detected Flt3 expression by AML blasts in more than 80% of patients, and exogenous Flt3-L altered blast proliferation in at least one *in vitro* model for a large majority of patients. The expression of Flt3 isoforms showed a wide quantitative variation between patients without there being any correlation with the effects of Flt3-L on blast proliferation, suggesting that downstream mechanisms are more important than receptor density for these functional Flt3-L effects.

Ligation of wild-type Flt3 leads to activation of the intracellular tyrosine kinase,[5] whereas abnormal Flt3 genes encode molecules with constitutive enzyme activity.[8,9] Our present results demonstrated that Flt3-L was a growth factor even for AML blasts with genetic Flt3 abnormalities, an observation suggesting that receptor ligation can induce additional growth-stimulatory signaling even in cells with constitutive kinase activity. Although it seems likely that this effect is caused by ligation of wild-type Flt3, the observation of increased proliferation in the presence of exogenous Flt3–L for Flt3–ITD$^+$ blasts without detectable wild-type suggests that ligation of mutated Flt3 may also contribute. However, this last observation should be interpreted with great care because relatively few patients were examined and the presence of small, undetectable amounts of wild-type Flt3 may influence these results. Furthermore, Flt3 ligation could also modulate colony formation and spontaneous apoptosis of *in vitro* cultured Flt3-ITD$^+$ and -ITD$^-$ blast populations. Our results thereby support the hypothesis that exogenous Flt3–L is an important growth factor for AML blasts in a major part of patients. In contrast, Flt3–L is an autocrine growth factor only for a small subset, and the importance of the autocrine secretion is also reduced by the presence of other exogenous cytokines (e.g. GM-CSF).

ITD$^+$ AML blasts have decreased proliferative capacity in stroma-supported cultures.[33,34] Our present study demonstrated an association between Flt3-ITD and decreased AML blast proliferation in suspension cultures. This observation suggests that the decreased proliferative capacity is caused by a direct effect of the Flt3-ITD on intracellular signaling and not by indirect effects with modulation of paracrine growth-regulatory mechanisms.

GM-CSF and SCF can increase AML blast proliferation for most patients,[37,38] but their effects on the proliferative capacity of native human AML blasts have not been compared with those of Flt3-L. All three growth factors had similar effects with regard to growth enhancement and anti-apoptosis and divergent effects on cytokine release (Figure 5),[37-40] including the autocrine growth factors IL1β, G-CSF and GM-CSF. Thus, the overall results demonstrate that these three cytokines have growth-enhancing effects that are only minimally influenced by local cytokine networks and are independent of autocrine growth factor release and regulation of apoptosis.

The growth-enhancing effect of Flt3-L was characterized in detail. Firstly, Flt3-L could not be detected in culture supernatants for any patient, but Flt3-L specific neutralizing antibodies inhibited spontaneous AML blast proliferation for two patients. This observation suggests that Flt3-L can function as an autocrine growth factor in a small minority of patients. Secondly, the growth-enhancing effects of Flt3-L were not due to increased IL1β release because (i) the Flt3-L effects on proliferation and IL1β levels showed no correlation; (ii) Flt3-L had divergent effects on cytokine-specific RNA levels both for IL1β and IL6 independently of its growth-enhancing effect; and (iii) Flt3-L enhanced proliferation even in the presence of the antagonistic IL1RA. Thirdly, the growth-enhancing effect of Flt3-L involved the clonogenic AML cell subset, and this effect could be detected when using various experimental approaches. Lastly, control experiments with serum-free conditions and enriched AML blast populations showed that the Flt3-L effects were not dependent on unidentified serum components or the small lymphocyte contamination.

The possible interaction between differentiation status and Flt3-L effects was investigated by various experimental approaches. Flt3-L had only minimal effects on AML blast differentiation in suspension cultures. Differentiation was then analyzed by light microscopy because we regard morphologic alterations as a more robust sign of differentiation than flow-cytometric detection of altered expression of a single or a few membrane molecules.[35,36,41-43] Flt3-L did not induce erythroid differentiation in the colony formation assays either. Thus, the effects of Flt3-L seem to be only minimally affected by and have only a minimal influence on the differentiation status of native human AML blasts.

To conclude, Flt3-L was able to modulate the functional phenotype of native human AML blasts, and the most important effect seemed to be enhanced proliferation. This growth-enhancing effect was observed both for AML cells with and those without genetic Flt3 abnormalities, and our results thereby suggest that intracellular signaling initiated by Flt3-ligation in the AML blasts is involved in leukemogenesis and possibly also chemosensitivity for a majority of patients.

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Pre-Publication Report & Outcomes of Peer Review

Contributions

All authors took part in the design of the study, interpretation of data and preparation of the manuscript. All authors approved the final version of the manuscript. The authors especially contributed to the following parts: ØB: collection of cells, investigation of AML blast proliferation, cytokine secretion and differentiation. RH: analysis of Flt3-internal tandem duplication. LW and BTG: analysis of apoptosis in AML blasts. TsH: analysis of cytokine RNA levels.

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Disclosures

Conflict of interest: none.
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Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Francesco Lo Coco, Deputy Editor. The final decision to accept this paper for publication was taken jointly by Professor Lo Coco and the Editors. Manuscript September 16, 2002; accepted February 25, 2003.

In the following paragraphs, the Deputy Editor summarizes the peer-review process and its outcomes.

What is already known on this topic

FLT3 signaling is increasingly relevant in AML, particularly after the discovery that constitutive activation of the receptor by gene mutation is common in this disease and may be targeted by recently developed inhibitors.

What this study adds

The study shows that FLT3 ligation initiates signaling and modulates AML phenotype irrespective of the FLT3 status (i.e. mutated or not).

Caveats

The study includes a selection of hyperleukocytic cases. It would be interesting to figure out whether similar observations are obtained in patients with low initial WBC counts.

Paper II

Molecular Cancer

BioMed Central

Research

Pre-apoptotic response to therapeutic DNA damage involves protein modulation of Mcl-1, Hdm2 and Flt3 in acute myeloid leukemia cells

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Abstract

Background: Acute myeloid leukemia (AML) cells are characterized by non-mutated *TP53*, high levels of Hdm2, and frequent mutation of the Flt3 receptor tyrosine kinase. The juxtamembrane mutation of *FLT3* is the strongest independent marker for disease relapse and is associated with elevated Bcl-2 protein and p53 hyper-phosphorylation in AML. DNA damage forms the basic mechanism of cancer cell eradication in current therapy of AML.

Hdm2 and pro-apoptotic Bcl-2 members are among the most intensely induced genes immediately after chemotherapy and Hdm2 is proposed a role in receptor tyrosine kinase regulation. Thus we examined the DNA damage related modulation of these proteins in relation to *FLT3* mutational status and induction of apoptosis.

Results: Within one hour after exposure to ionizing radiation (IR), the AML cells (NB4, MV4-11, HL-60, primary AML cells) showed an increase in Flt3 protein independent of mRNA levels, while the Hdm2 protein decreased. The *FLT3* mutant MV4-11 cells were resistant to IR accompanied by presence of both Mcl-1 and Hdm2 protein three hours after IR. In contrast, the *FLT3* wild type NB4 cells responded to IR with apoptosis and pre-apoptotic Mcl-1 down regulation. Daunorubicin (DNR) induced continuing down regulation of Hdm2 and Mcl-1 in both cell lines followed by apoptosis.

Conclusion: Both IR and DNR treatment resulted in concerted protein modulations of Mcl-1, Hdm2 and Flt3. Cell death induction was associated with persistent attenuation of Mcl-1 and Hdm2. These observations suggest that defining the pathway(s) modulating Flt3, Hdm2 and Mcl-1 may propose new strategies to optimize therapy for the relapse prone *FLT3* mutated AML patients.

Background

Anthracyclines like daunorubicin (DNR) are used in the induction treatment of acute myeloid leukemia (AML), obtaining short time complete hematological remission for more than 65% of adult AML patients with *de novo* AML [1]. Successful hematological remission after only one induction cycle is a favorable prognostic parameter and is associated with decreased risk of later AML relapse [1,2]. Induction therapy causes rapid activation of the tumor suppressor p53 followed by dominating p53-targeted gene expression *in vivo* [3]. A major mechanism for this p53 induction is DNA damage through anthracycline-stabilization of the DNA:topoisomerase II complex [4], but cell death induction by anthracyclines may also involve other molecular targets independent of p53 [4-7].

Ionizing radiation (IR) is frequently used in the treatment of solid cancers, in the conditional treatment before allotransplantation of leukemia patients and in radioisotope-conjugated therapeutic antibodies directed against AML cells [8,9]. IR and anthracyclines induce growth arrest and cell death through DNA-damage, but also involve cell membrane-related effects in regulation of apoptosis [4-7,10]. We have previously reported that AML patient cells respond with varying sensitivity to IR-induced proliferation arrest [11], and it may therefore be of interest to determine molecular mechanisms for radioresistance in more detail.

The strongest molecular predictor for AML relapse is internal tandem duplications in the juxtamembrane domain of the receptor tyrosine kinase Flt3 (Flt3-ITD). These mutations are present in approximately one third of the patients [12]. Flt3-ITD are associated with increased DNA repair [13], an observation suggesting that these cells are able to recover from DNA damage caused by topoisomerase II blockage and thus have a more drug-resistant phenotype. The expression of anti-apoptotic Bcl-2 protein family members is also influenced by the mutational status of Flt3 [14]. We have recently shown that a duplication of Y591 in Flt3-ITDs is associated with elevated Bcl-2 protein and hyper-phosphorylated wild type (wt) p53 in AML, proposing a mechanism for inactivation of p53 [14].

Mcl-1 is an anti-apoptotic member of the Bcl-2 family of proteins. High levels of Mcl-1 have been detected in cells from patients with relapsed AML [15]. Therapeutic targeting of Bcl-2 family proteins seems to depend on Mcl-1 to trigger apoptosis [16]. It may therefore be of particular interest to examine the Mcl-1 modulation in DNA damage therapy.

In contrast to solid tumors, more than 90% of the AML cases comprise wild type p53 [17,18]. On the other hand, the E3 ubiquitin ligase Hdm2 is usually strongly expressed in AML, contributing to block the growth inhibitory and pro-apoptotic effect of p53 [19]. IR induces DNA damage and rapid down regulation of Hdm2 through induction of auto-ubiquitination and subsequent proteasomal degradation [20]. Recent reports indicate that Hdm2 have important p53-independent activities, including regulation of cell membrane receptors like insulin-like growth factor (IGF) 1 receptor and β2-adrenergic receptor through ubiquitination [21]. However, it is not known whether the Flt3 receptor is regulated by Hdm2.

Concerted protein modulation of a receptor tyrosine kinase, the E3 ubiquitin ligase Hdm2 and selected Bcl-2 family members through DNA damage therapy has previously not been reported. Our study indicated that both IR and DNR induced Hdm2 protein down regulation, partly Flt3 protein elevation, and a pro-apoptotic shift in the expression of proteins in the Bcl-2 family. Flt3 and Hdm2 might have a reciprocal regulation at the protein level and *FLT3* mutations could be involved in protection against IR-induced apoptosis through a persisting Mcl-1 level.

Results

Ionizing radiation induces reciprocal regulation of Flt3 and Hdm2 protein in NB4 cells

The promyelocytic cell line NB4 is characterized by mutated *TP53* and non-functional p53 protein [22,23] as well as wild type *FLT3* [24]. DNA damaging 25 Gy IR of NB4 cells resulted in increased apoptosis, but no modulation of *FLT3* or *HDM2* mRNA was observed (Fig. 1a,b; left panel). Hdm2 responds to IR with protein auto-degradation [20], and it regulates endocytosis of certain receptors like the IGF 1 receptor [25]. We examined Flt3 and Hdm2 at different time points after IR (25 Gy) and found highly significant reciprocal regulation at the protein level (Fig. 1c; left panel). This was accompanied by attenuation of the anti-apoptotic Mcl-1, an increase in Bax but unaltered Bcl-2 (Fig. 1d; left panel) and Bcl-X_L (data not shown). Previous studies have shown that DNA-damaging *in vivo* chemotherapy of AML has no effect on *MCL-1* gene induction, but rapidly induces *BAX* and *PUMA* mRNA [3] (Øyan et al., manuscript in preparation). p21 protein was not detected in NB4 cells (data not shown), and the p53 protein level was not altered after irradiation (Fig. 1d; left panel), reflecting its non-functional status.

Hdm2 response and stable Mcl-1 in the IR-resistant cell line MV4-11

MV4-11 is characterized by *FLT3-ITD*, loss of wilt type *FLT3* allele, and wild type *TP53* [22,24]. MV4-11 cells were resistant to IR with regards to apoptosis induction (Fig 1a, right panel), but responded with more than one fold increase in *HDM2* mRNA (Fig. 1b), reflecting the functional p53. The level of Hdm2 protein showed a

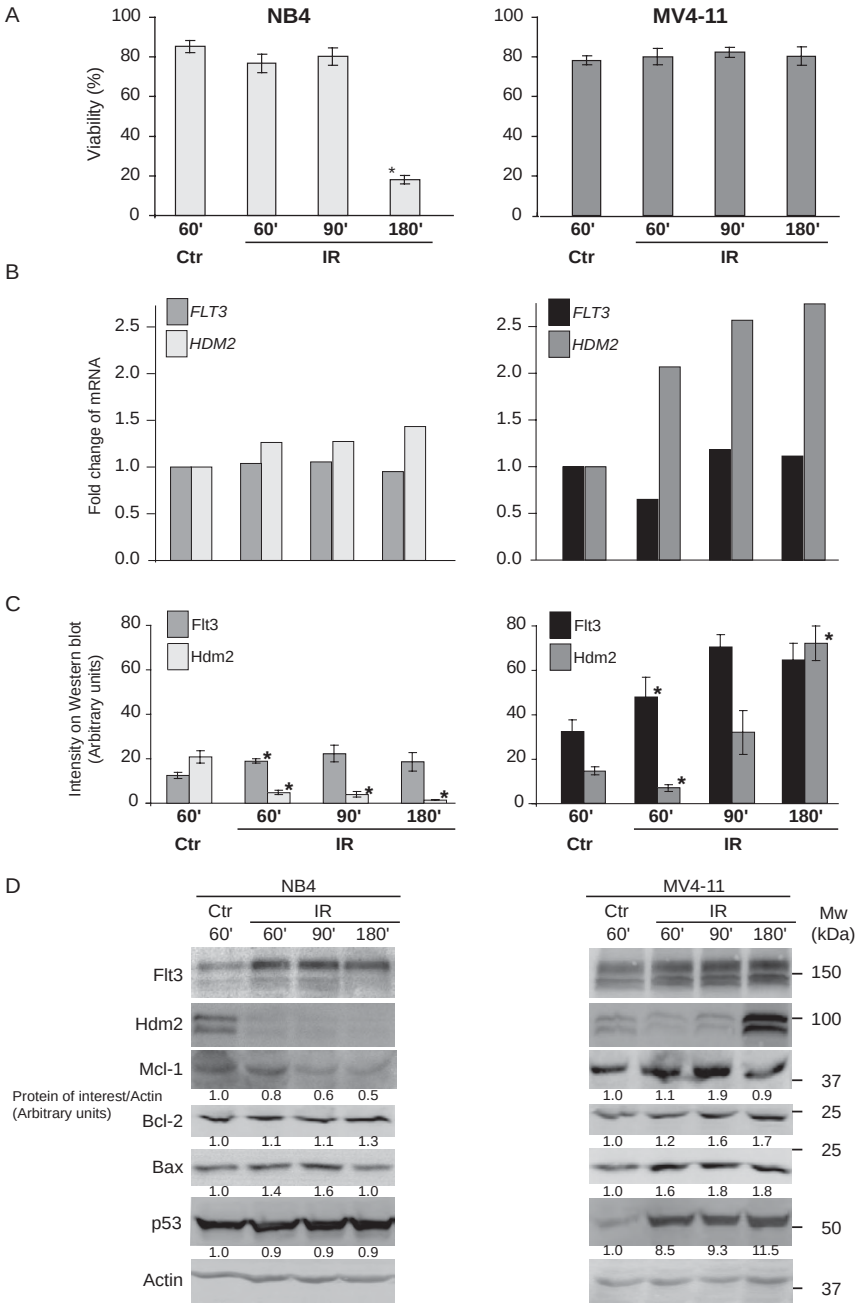

Figure 1

Rapid IR-induced protein modulation of Flt3, Hdm2 and Bcl-2 family members in AML cell lines. **A**. Cells were exposed to 25 Gy and fixed after the indicated time (minutes). The percentage of normal nuclei in a total of 200 cells was determined in Hoechst stained cells for each time point. The results shown represent the mean of three separate experiments and the error bars show standard error of mean (SEM = Standard Deviation/√n). The star denotes statistical significance relative to the control, this is determined by a Students two-tailed *t*-test, $p < 0.05$. **B**. mRNA level of *FLT3* and *HDM2* was determined by Real-time PCR in one typical experiment. GAPDH was used as endogenous control. **C**. IR down regulated Hdm2 protein and up regulated Flt3 protein in these AML cell lines. The diagram shows measured intensity on three separate Western blots (normalized to Actin). The error bars show standard error of mean SEM and the stars represents significance as in **A**. **D**. Visualization of the protein modulations of Flt2 and Hdm2 shown in **C**. in addition to modulation of proteins in the Bcl-2 family. Mean intensity on the Western blots written below the corresponding panel were measured and normalized to Actin and to the control. The values shown are arbitrary units and represent one typical experiment.

small but significant decrease after 60 minutes before an increase was detected, whereas the Flt3 level increased in response to IR and was not attenuated by the elevated *HDM2* level after 180 minutes (Fig. 1c,d). Another striking difference from the NB4 cells with *FLT3-wt* was that the Mcl-1 level did not change in response to IR (Fig. 1d). Furthermore, MV4-11 responded to IR with increased protein levels of p53, Bax, Bcl-2, (Fig. 1d) and p21 (data not shown) while the level of Bcl-X_L was unaltered (data not shown). The IR induction of p53, Hdm2, Bax and p21 suggests that the p53 transcriptional activation in MV4-11 is intact [3].

Attenuation of Hdm2 and Mcl-1 is independent of p53 and Flt3

The effect of IR was also examined in HL-60 cells, characterized by wild type *FLT3* and deleted alleles for *TP53* [22,24]. Like inNB4 and MV4-11 cells, Hdm2 was attenuated and Flt3 increased, but the Flt3 protein appeared not to be full length (Fig. 2a; ~150 versus ~60 kDa). The lack of full length Flt3 was confirmed in cells from both ATCC and DSMZ within four passages of culture, and immunoprecipitation of Flt3 in these cells did not reveal any low molecular anti-Flt3 reactive form (data not shown). Flt3 protein has previously been reported non-detectable in HL-60 cells [26].

Bcl-2 family members showed no significant response to IR in HL-60 cells except a late decrease in Mcl-1.

Human primary AML cells (patient 1) were irradiated and examined for Flt3 and Hdm2 modulation (Fig. 2b), indicating that the reciprocal Flt3-Hdm2 response to DNA damage also could be present in primary leukemia cells. In contrast to the HL-60 cells where the p21 response was absent, early increase was present in the primary AML cells. These differences reflects an absence of a p53 response in HL-60 cells and a presence of such in the patient cells (Fig. 2a,b).

Daunorubicin induces attenuation of Hdm2 and Mcl-1 independent of TP53 *and* FLT3 *status*

Since both DNR and IR induce DNA damage, we examined the effect of DNR in both AML cell lines (NB4, MV4-11 and HL-60) and primary cells (patient 2). The cells were treated *in vitro* (Fig. 3) with DNR for 5 hours at relevant concentrations [3]. The NB4 and MV4-11 cell lines were sensitive to DNR with regards to apoptosis induction, and both Mcl-1 and Hdm2 were down regulated (Fig. 3a). Although DNR increased Flt3 protein in all the AML cells tested (Fig. 3ab), this effect was most prominent in MV4-11 cells, HL-60 and the primary AML cells. HL-60 cells showed an increase in putative short forms of Flt3 protein with low doses of DNR (Fig. 3b).

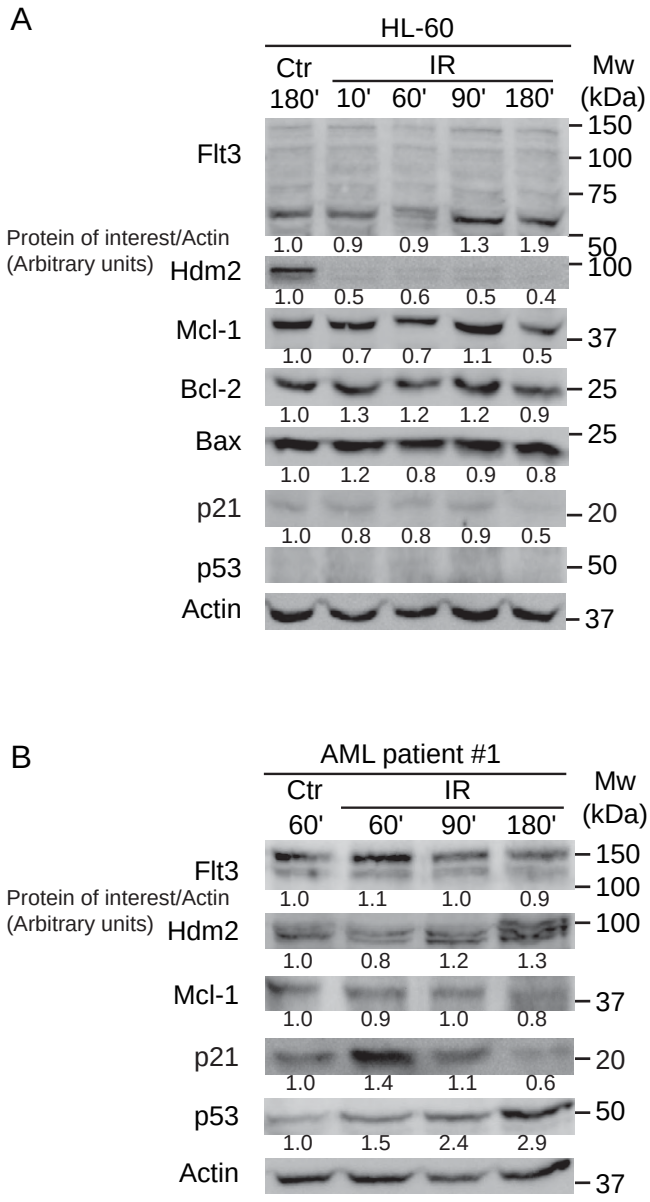

Figure 2

IR decrease Hdm2 protein in HL-60 (p53 -/-) and in primary AML cells. **A**. HL-60 cells treated with 25 Gy and harvested at indicated time (minutes) demonstrated a rapid decrease in Hdm2 protein after IR. There was no detectable Flt3 protein at 130 kDa. **B**. Primary AML cells also treated with IR and harvested at indicated time, demonstrated the rapid Flt3 increase and Hdm2 attenuation as previously observed. This was followed by an increase in Hdm2, reflecting functional and elevated p53 protein. Mean intensity on the Western blots were measured and normalized to Actin and to the control. Values shown are arbitrary units and represent one typical experiment.

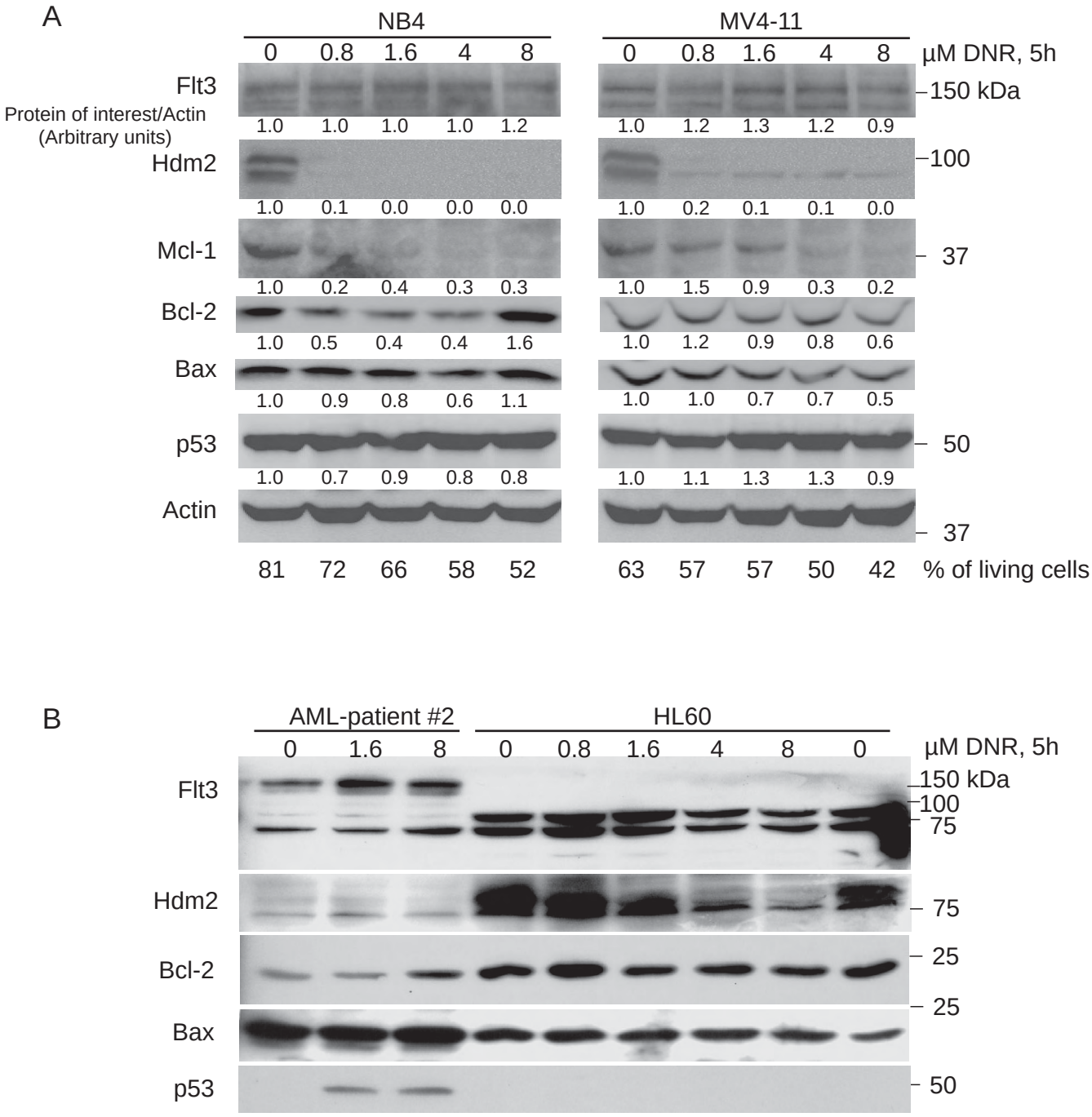

Figure 3

Daunorubicin therapy of primary AML cells and cell lines increased Flt3 protein and attenuated Hdm2/Mcl-1. **A**. Treatment of NB4 and MV4-11 cells with DNR resulted in an increase in Flt3 and a decrease in Hdm2. The mean intensity on one representative Western blot was calculated and normalized to Actin and to the control. The numbers shown are in arbitrary units and represent one typical experiment. The percentage of living cells was determined by flow cytometry. The living cells distinct forward and side scatter properties were used to separate viable cells from dead cells. **B**. Increasing doses of DNR induce Flt3 and down regulate Hdm2 protein in primary AML cells *in vitro*. Note that Hdm2 is down regulated in HL-60 cells, a cell line with lack of full length Flt3 protein and with deleted alleles for p53. All wells on the SDS-PAGE gel was loaded with equal amounts of protein, and Coomassie staining of the gel after blotting confirmed this equal loading.

Flt3 and Hdm2 protein are reciprocally regulated in vivo

We have recently demonstrated swift induction of p53 and Bax proteins in AML cells collected from patients undergoing induction chemotherapy with anthracyclines and cytarabine [3]. It was therefore of interest to examine the AML cells' protein levels of Flt3 and Hdm2 after *in vivo* chemotherapy (Fig. 4, one representative patient). AML cells from patients were collected within the first 4 hours of chemotherapy, and showed a strong *in vivo* decrease in Hdm2, in addition to increase in p53 and Flt3.

Discussion

We demonstrated that Flt3 protein increased in response to IR and DNR in all AML cell lines and primary leukemic cells tested, *in vitro* and *in vivo*. Likewise, Hdm2 was down regulated in concert with the Flt3 increase. This reciprocal regulation was consistent in all experiments except in MV4-11 cells 3 hours after IR and in NB4 cells treated with DNR. A summary of all the results is shown in Fig. 5. Several scenarios may explain this mutual modulation of Flt3 and Hdm2. The IGF 1 receptor, a more distant relative of Flt3, has been demonstrated to undergo Hdm2-dependent ubiquitination and degradation [25]. If Hdm2 regulates the turnover of Flt3 through ubiquitination, IR-induced Hdm2 degradation will result in elevated levels of Flt3. The observed down regulated Hdm2 in irradiated AML cells (Fig. 5) is probably due to proteasomal degradation [27,28]. In addition to its ability of auto-ubiquitination, Hdm2 is ubiquitinated by several E3 ubiquitin ligases, including the p300-CBP associated factor (PCAF) and TSG101 [29,30]. Future work is needed to address if modulation of Flt3 level may affect the level of Hdm2, and if this possible action is directly mediated by Flt3 on Hdm2 or involves other E3 ligases.

It can not be ruled out that the increase in Flt3 protein after IR is based on mechanisms independent of Hdm2. IR has been shown to increase the mRNA and protein lev-

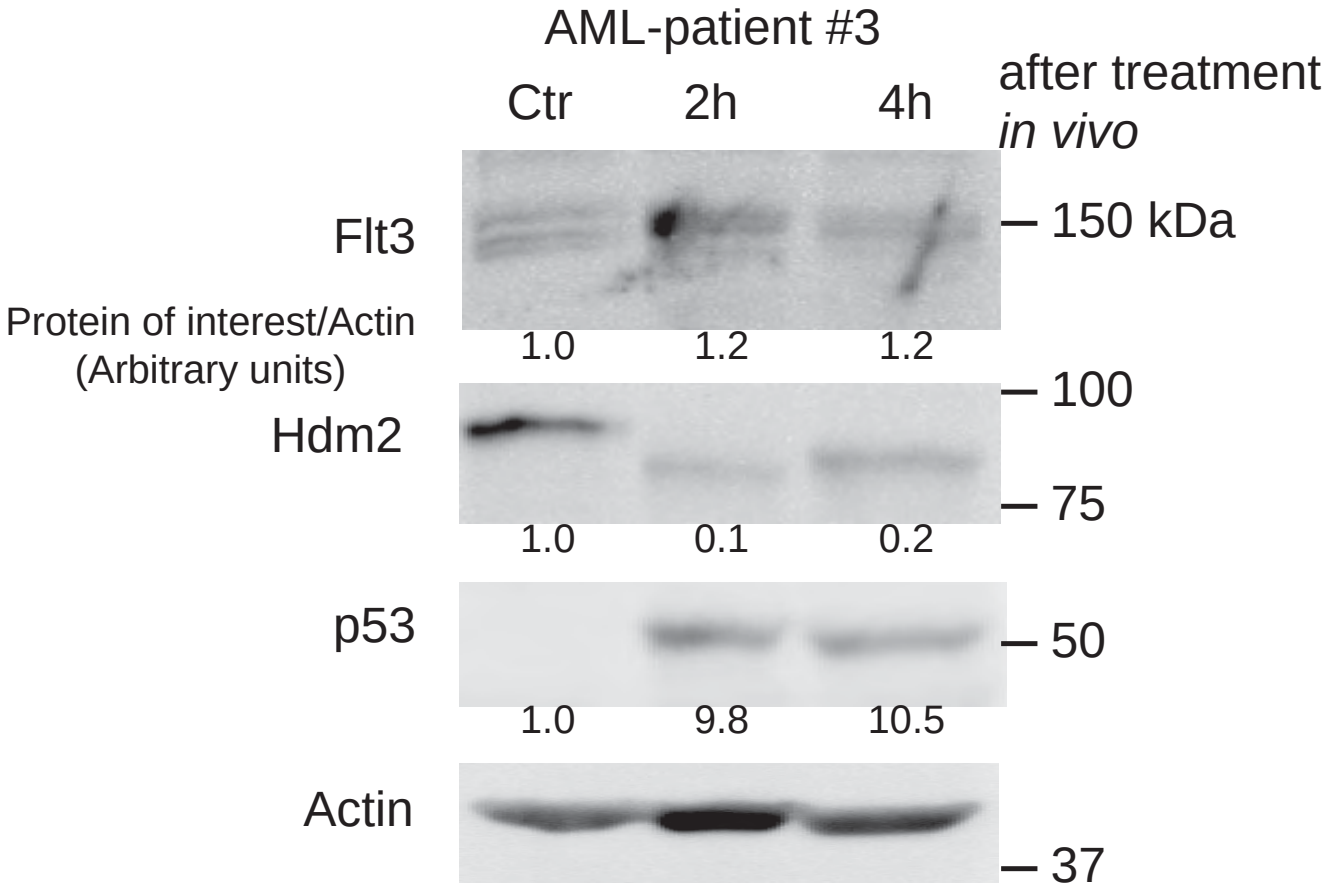

Figure 4

Induction therapy of AML reciprocally regulates Flt3 and Hdm2 proteins *in vivo*. AML cells sampled from a patient undergoing induction chemotherapy with an anthracycline and cytarabin were subjected to Western blotting and analyzed for Flt3, Hdm2 and p53 expression. The mean intensity on one representative Western blot was calculated and normalized to Actin. The numbers shown are in arbitrary units.

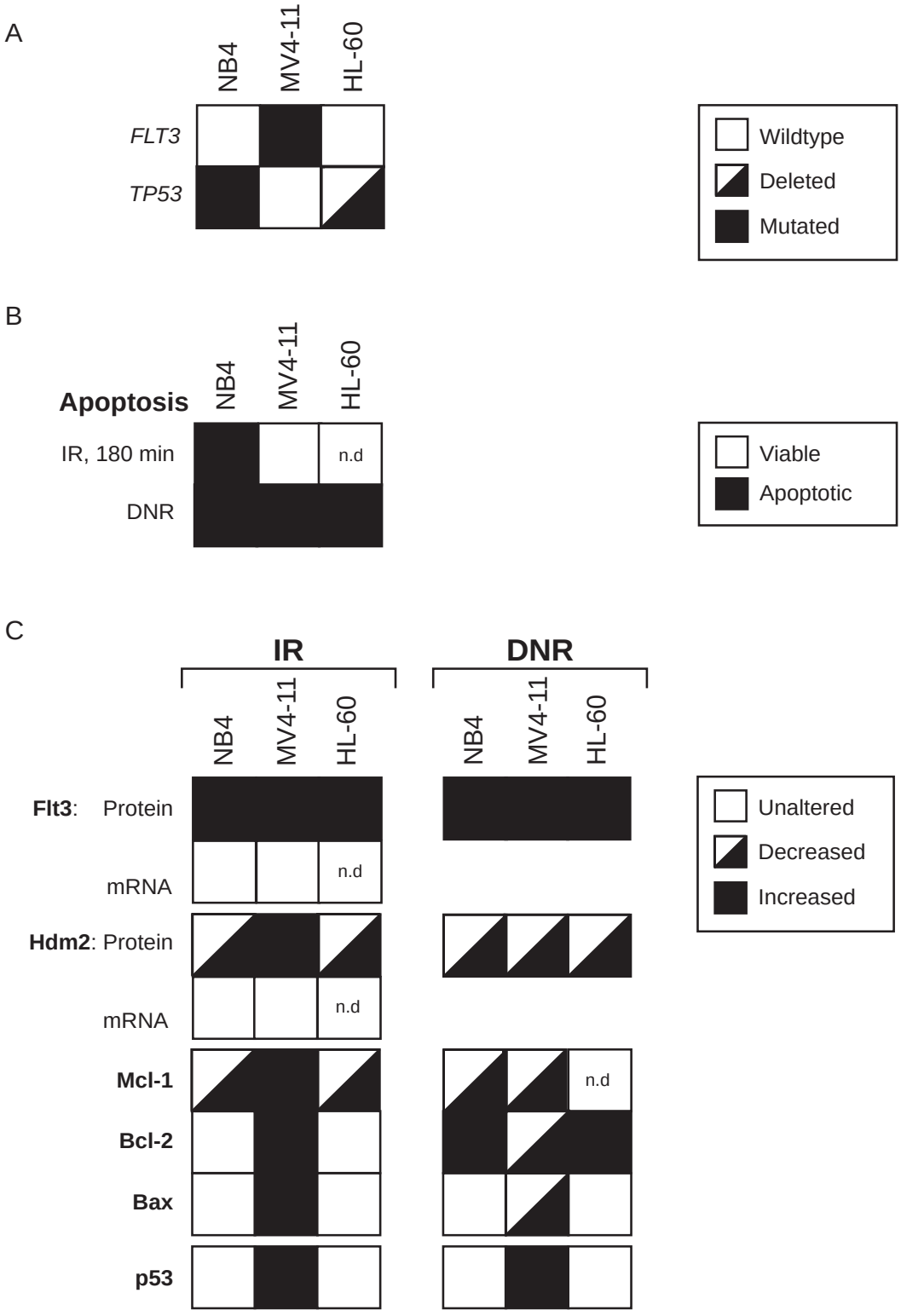

Figure 5

Summary of the results. **A**. *FLT3* and *TP53* mutational status of the cell lines used in this study. **B**. A summary of the ability of IR and DNR to induce apoptosis in the three different cell lines studied (n.d; not determined). **C**. Overview of the concerted protein modulations elicited by DNA-damaging therapy found in this study (n.d; not determined).

els of epidermal growth factor (EGF) receptor as well as the cell surface protein expression of IGF 1 receptor [31,32]. Such mRNA regulation of Flt3 after IR was not observed in our study (Fig. 5).

The NB4 cells, in contrast to the MV4-11 cells, showed IR induced apoptosis (Fig. 5) and a lack of increase in *HDM2* mRNA level. Since NB4 cells have non-functional p53 [33], this suggests that NB4 undergoes a p53-independent apoptosis during IR-exposure. A possible explanation for the IR-resistance of MV4-11 is that AML cells with Flt3-ITD can repair double-stranded breaks in DNA more efficient than in cells with wild type Flt3 [13], but an anti-apoptotic effect on p53 by the MLL-fusion products may be an alternative mechanism [34]. This makes MV4-11 more protected against apoptosis induced by IR. Other explanations for early IR-induced apoptosis in NB4 cells in contrast to in the MV4-11 cells could include a pro-apoptotic response on the Bcl-2 family members and a lack of Hdm2 induction (Fig. 5). No shift in the balance of Bcl-2/Bax was observed (Fig. 5), thus our data suggest that Mcl-1 is a central player in regulation of DNA-damage induced cell death. A striking feature of IR treated NB4 cells, as well as DNR treated NB4 and MV4-11 cells, was the Mcl-1 down regulation accompanied by apoptosis. These observations emphasize the putative importance of Mcl-1 in regulation of apoptosis in AML, with possible implications for the biology behind disease relapse [15,16].

MV4-11 cells were resistant to IR while DNR effectively induced apoptosis (Fig. 5). DNR elicited a lasting Hdm2 and Mcl-1 down regulation in contrast to IR. This suggests that DNR ignites apoptosis through more pathways than IR and that the Mcl-1 attenuation is a pre-apoptotic event. In addition to the induction of DNA damage, DNR is known to stimulate the level of the second messenger ceramide by *de novo* synthesis and thus trigger apoptosis [5]. Anthracyclines may also induce apoptosis via signalling through altered plasma membrane lipid rafts and the death receptor pathway [6] (for review see [7]).

The p53-deficient HL-60 cell line demonstrated Hdm2 decrease as well as a putative Flt3 increase in response to IR or DNR (Fig. 5). The *FLT3* gene in HL-60 is wild type [24], confirmed by sequencing of the juxtamembrane region and the kinase activation domain. Interestingly, lack of full length Flt3 protein in HL-60 has previously been reported [26], and we were not able to detect full length Flt3 in different batches of HL-60 cells from ATCC and DMZS (Fig. 3b). The protein bands between 50 and 100 kDa may be protein products from alternative splicing of *FLT3* mRNA, as reported for the closely related platelet-derived growth factor alpha-receptor and *KIT* [35,36]. Additional work is clearly needed to address the possibility of alternative splicing of *FLT3* in HL-60 and in AML cells in general.

Flt3-ITD is the strongest predictor for relapse of AML in therapy with anthracyclines [12], and is recently associated with enhanced DNA repair [13]. We demonstrated that the anti-apoptotic protein Bcl-2 was induced in MV4-11, HL-60 cells and primary AML cells during DNA damage therapy (Fig. 5). This could indicate that anthracyclines elicit an anti-apoptotic signal through Flt3. The anti-apoptotic signal may be particular strong in AML cells with a Flt3-ITD mutation including an Y591 duplication [14].

Conclusion

In this study we show a concerted protein modulation of Flt3, Hdm2 and Mcl-1 after DNA damaging therapy in AML. IR resulted in decreased levels of Hdm2 and elevated levels of Flt3 and may involve p53 independent activities of Hdm2 acting on Flt3 as proposed for other receptor tyrosine kinases. The apoptotic response may depend on a persisting down regulation of Hdm2 and Mcl-1 [37]. Targeting of Flt3, Bcl-2/Bcl-X_L and Mcl-1 is proposed to enhance the response of chemotherapy. Preclinical studies and early clinical trials that follow these principles are underway [38,39], and we believe that relevant biomarker examinations [3] including the proteins presented in this study may help to pinpoint the patients that will benefit from this enhanced therapy.

Methods

Cell culture

All patient studies were approved by the local ethical committee (REK Vest) and the Data Inspectorate, Norway. REK Vest is affiliated with the University of Bergen and Haukeland University Hospital. Samples were collected after informed consent. Patient data is overviewed in Table 1.

Leukemic peripheral blood mononuclear cells (PBMC) were isolated by density gradient separation (Ficoll-Hypaque; Nycomed, Oslo, Norway) and were stored frozen in liquid nitrogen [40]. The percentage of blasts among leukemic PBMC exceeded 95% for all patients as judged by light microscopy of May-Grünwald-Giemsa stained cytospin smears [41]. PBMC were cultured in serum free conditions in StemSpan (Stem Cell Technologies, Vancouver, BC, Canada) at an average concentration of 2×10^6 cells per ml. Cells collected from patients during therapy followed the procedures as described by Anensen et al. 2006 [3].

The AML cell line NB4, kindly provided by Dr. Michel Lanotte (INSERM U-301, Hôpital St. Louis, Centre Hayem, Paris, France) [42], was cultured in RPMI 1640

Table 1: Clinical and biological characteristics of AML patients

Patients	Age	Sex	Previous malignant disease	FAB	Membrane molecules					Karyotype	*FLT3 LM*	*FLT3 Asp835*	Survival (Weeks)
					CD 13	CD 14	CD 15	CD 33	CD 34				
# 1	72	M	Residive	AML M1	+	-	+	+	-	Normal	wt	wt	6
# 2	34	F	-	AML M5a	-	-	+	+	-	46 XX, t(9;11), (q22;q23)	wt	0.31	>24 (Tx)
# 3	55	M	-	Atypical	+	-	+	+	-	Multiple	wt	wt	>23

The patients were randomly selected; their clinical and biological data are included as background information.

(Sigma-Aldrich, Inc. St. Louis, MO, USA) with 10% fetal bovine serum (Foetal Calf Serum Gold, PAA Laboratories GmbH, Pasching, Austria) and penicillin/streptomycin 50 IU/50 μg per ml. Sequence analysis of both DNA strands of the NB4 cells used in this study confirmed wild type juxtamembrane region and activation loop of *FLT3*, and FISH analysis confirmed the presence of t(15;17) translocation. The same culture conditions as for NB4 were used for HL-60, purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). Reverse transcriptase PCR of HL-60 confirmed presence of normal length of *FLT3* mRNA in the juxtamembranous region. The MV4-11 cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA) and cultured in IMDM (BioWhittaker, Cambrex Bio Science, Verviers, Belgium) with 10% FBS and penicillin/streptomycin 50 IU/50 μg per ml. The *FLT3* gene in MV4-11 comprised a length mutation in the juxtamembrane region, and the t(4;11)(q21;q23) translocation was confirmed by FISH. The *TP53* gene in MV4-11 is wild type according to data published [22] and the IARC *TP53* Database [43]. The protein level of Flt3 in NB4 was approximately 50% of the level in MV4-11, estimated by Western blot and flow cytometry.

Irradiation and chemotherapy treatment of cells

For irradiation induced DNA double strand breaks, samples were exposed to 25 Gray (Gy) from a Ce^{137} source [11] and maintained in culture until samples were collected for Western blot analysis at time indicated. To secure that the observed effect was from the irradiation, the control samples were handled the same way as the exposed samples except for the actual irradiation. Collection of cells from AML patients under therapy and *in vitro* treatment of cells with daunorubicin was performed as previously described [3].

Apoptosis assays

Cells were fixed in 2% paraformaldehyde solution containing the DNA specific nuclear stain Hoechst (Hoechst 33342, Invitrogen, Carlsbad, CA, USA; 10 μg/ml) and examined as previously described [33]. The number of normal and apoptotic nuclei was counted in an inverse fluorescence microscope (×400 magnification; Leica IRB, Leica Microsystems GmbH, Wetzlar, Germany). The mean number of three experiments was calculated together with the standard error of mean (standard deviation/√number of experiments). Nuclear staining with Hoechst of the cells treated with daunorubicin was not possible due to the strong fluorescence from the drug. These cells were fixed in 4% paraformaldehyde solution and their forward scatter and side scatter properties were examined by flow cytometry and used to determine the number of living cells. Flow cytometry was performed on a FacsCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and data analyses were carried out using the FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Western blotting

Samples for Western blotting were prepared by pelleting the cells (3–10 millions) and washing them twice in 0.9% NaCl following lysis in the following buffer: 10 mM Tris (pH 7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP40, 5 mM NaF, 0.5 mM sodium orthovanadate, 1 mM DTT, and 0.1 mM PMSF (50–200 μl lysis buffer per sample) and transfered to 1.5 ml tubes. The samples were homogenized by 20 strokes of a plastic mini homogenizer before centrifugation at 14000 × g for 20 minutes. Protein concentrations were determined using the Bradford protein assay, following the manufacturers instructions (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein samples were added SDS loading buffer (Final: 1% SDS, 10% Glycerol, 12 mM Tris-HCl pH 6.8, 50 mM DTT and 0.1% Bromophenol Blue) and boiled for 10 minutes.

SDS-polyacrylamide gels, 10 or 12.5 % were loaded with 50–70 μg protein per well. After electrophoresis (100–200 V, 1–3 hours) and electroblotting (200 mA, o/n 4°C) the PVDF-membranes (HybondP, Amersham Biosciences, Oslo, Norway) were blocked for 1 hour in I-Block Blocking agent (Applied Biosystems, Foster City, CA, USA). Primary antibodies were incubated for 1–2 hours in room temperature or over night at 4°C followed by 1 hour washing in TBS-Tween. The antibodies Flt3 S-18, Hdm2 SMP-14, p53 BP53-12, Mcl-1 22, Bcl-2 ΔC 21 and Bax 2D2 were from Santa Cruz Biotechnology, CA, the Actin

antibody AC-15 was from Abcam plc, Cambridge, UK and the Hdm2 antibodies 2A10 and IF2 were from Calbiochem, San Diego, CA, USA.

Secondary antibodies conjugated to horse radish peroxidase (Jackson ImmunoResearch laboratories, West Grove, PA, USA) were diluted in 4% fat-free dry milk in TBS-Tween and incubated 1 hour at room temperature. After washing for 1 hour with TBS-Tween, the membranes were developed using Supersignal® West Pico or West Femto Chemiluminiscence Substate from Pierce Biotechnology Inc, Rockford, IL, USA according to the manufacturers' instructions. The membranes were imaged using a Kodak Image Station 2000R (Eastman Kodak Co., Lake Avenue, Rochester, NY, USA), and bands were quantified using the Kodak analysis software. Data were exported to Excel spreadsheet, corrected for background and loading control intensities and a Student's two-tailed *t* test was used for determination of significance.

Real time PCR

Immediately after *in vitro* experiments, 5×10^6 cells were dissolved in RNAlater (Ambion Inc.) to stabilize and protect RNA and then stored at -80°C. RNAeasy plus mini kit (Qiagen Inc.) was used for isolation of total RNA. Cells were thawed, centrifuged and resuspended in RTL buffer and further procedures were followed according to manufacturer's instructions. RNA quality was tested on a 2100 Bioanalyzer (Agilent Technologies) and total RNA was quantified with a spectrophotometer for small aliquots (NanoDrop Technologies, Wilmington, DE, USA). cDNA were synthesized using the High-Capasity cDNA Archive Kit (Applied Biosystems, Foster City, CA) running 625 ng RNA in 50 μl total reaction volume. Real Time PCR was performed using assays-on-demand containing primers and FAM dye-labelled probes. Human GAPDH and β-Actin were used as endogenous controls. For Flt3 and Hdm2, assays Hs00174690_m1 and Hs00234753_m1 (Applied Biosystems) were used. TaqMan Universal PCR Master Mix (Applied Biosystems) was run with 2 μl cDNA in 10 μl total reaction volumes. The PCR was performed in a 384-well clear optical reaction plate on a 7900HT real time PCR system (Applied Biosystems). The calibrator sample in each experiment was used for standard curve dilution. All samples were run in three replicates and data were analyzed using the relative standard curve method as described by the manufacturer (Applied Biosystems).

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

LW participated in the study design, performed experiments leading to Figure 1, 3 and 4 and wrote the manuscript. GS carried out the real-time PCR. IH contributed with the experiments in Figure 2. RH performed the sequencing of Flt3 and cytogenetics in patient cells and cell lines. ØB collected the patient material and participated in the design of the study. BTG conceived the study, participated in the study design and wrote the manuscript. All authors participated in the finalization of the manuscript, and read and approved the final manuscript for submission.

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Paper III

Flt3 mutations proximate to its ubiquitin dependent endocytosis motif suspend Hdm2 modulation

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Abstract

Purpose: Acute myeloid leukemia (AML) frequently features mutations in the receptor tyrosine kinase Flt3 and elevated expression of the oncogenic E3 ubiquitin ligase Hdm2. Additional to the p53 inhibitory effect of Hdm2, Hdm2 appears involved in endocytosis of cell surface receptors. In this study we explore the possibility of Flt3 modulation by Hdm2.

Experimental design: Primary AML cells and cell lines (NB4 and MV4-11) with wild type Flt3 (Flt3-wt) or mutated Flt3 (Flt3-ITD) were used with Flt3 ligand (FL), small molecular inhibitors and small interfering RNA (siRNA) to elucidate the relation between Flt3 and Hdm2 on protein level, mRNA expression and modulation of apoptosis.

Results: The basal level of Flt3 is higher in AML patients with Flt3-ITD than in patients with Flt3-wt. Flt3-ITD affects an ubiquitin dependent endocytosis motif possibly resulting in enhanced receptor cycling. Down-regulation of Flt3-wt by FL, small interfering RNA or PKC412 resulted in elevated level of Hdm2. Similarly, Hdm2 attenuation resulted in increased Flt3 protein expression. Flt3-ITD responded less to Flt3 down-regulation, and was only weakly responding to Hdm2 modulation.

Conclusion: We demonstrate that modulation of Flt3 or Hdm2 results in reciprocal regulation, and that Flt3 with internal tandem duplications may suspend its Hdm2 modulation. Together, Flt3-ITD results in dysregulated receptor turnover and an attenuated mechanism for Hdm2 down-regulation. Thus Hdm2 is interconnecting the two pathways of Flt3 and p53, both related to chemoresistance in AML.

Paper IV

Bcl-2 protects against p53-induced apoptosis through Hdm2

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Abstract. Hdm2 is up-regulated in several malignancies including sarcomas and acute myeloid leukemia, where it counteracts the anti-proliferative and pro-apoptotic effect of wild type p53. The anti-apoptotic protein Bcl-2 is often elevated in many tumors with wild type p53, and serves to block p53-induced apoptosis. We demonstrate that the protein level of Hdm2 protein positively correlates with the level of Bcl-2, following Bcl-2 levels in different cells and tracking Bcl-2 modulated by over-expression or shRNA knockdown. Consequently, treatment with the Bcl-2 small inhibitory molecule HA14-1 attenuated the level of Hdm2. The Bcl-2 level, but not the DNA damage induced Hdm2 degradation, was affected by the disrupted E3 ubiquitin ligase activity of Hdm2. DNA-damage induced Hdm2 down-regulation was blocked by disrupted E1 activity, defect polyubiquitination and proteasome inhibitors. Finally, we show that Bcl-2 protection from p53-induced cell death requires co-expression of Hdm2 in double null p53/mdm2 mouse embryonic fibroblasts. Our results indicate that Bcl-2 regulates Hdm2 level, and that Hdm2 is a key mediator in Bcl-2 inhibition of p53-induced apoptosis. This is of particular therapeutic interest for cancers displaying elevated Hdm2 and Bcl-2.