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Dissecting the role of the cytoplasmic mutant Nucleophosmin in acute myeloid leukaemia development

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1. List of abbreviations

5FU	5-fluoruracile
APL	Acute Promyelocytic Leukaemia
APAAP	Alkaline Phosphatase Anti-Alkaline Phosphatase
AML	Acue Myeloid Leukemia
ALCL	Anaplastic Large-Cell Lymphoma
BM	Bone Marrow
BMT	Bone Marrow Transplatation
BrdU	5-bromo-2'-deoxyuridine
CSC	Cancer Stem Cells
CAD	Caspase Activated DNase
CEBPA	CCAAT/Enhancer Binding Protein gene
CLPs	Common Lymphocyte Progenitors
CMP	Common Myeloid Progenitors
EIF2	Eukaryotic translation Initiation Factor 2
FLT3-ITD	Internal Tandem Duplication of Fms-related Tyrosine kinase 3 gene
FAB	French-American-British
GMPs	Granulocyte-Macrophage Progenitors
HSC	Haematopoietic Stem Cells
HSPC	Haematopoietic Stem Progenitor Cells
LKS	Lin-, cKit+, Sca+
LT-HSCs	Long Term haematopoietic stem cells
MDS	MyeloDysplastic Syndrome

MEPs	Megakaryocyte-Erythrocytes Progenitors
MLL	Mixed-Lineage Leukaemia gene
MPD	Myelo Proliferative Disease
MPP	MultiPotent Progenitor cells
NK	natural killer
NK	normal karyotype
NPM	Nucleophosmin
NES	Nuclear Export Signal
NLS	Nuclear Localization Signal
NoLS	Nucleolar Localization Signal
PTDs	Partial Tandem Duplication
PKR	RNA-dependent Protein Kinase
ST-HSCs	Short Term
HSPC	Stem/Progenitor Cell
TKD	Tyrosine Kinase Domain
WHO	World Health Organization

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3. Abstract

Acute myeloid leukaemia (AML) is a genetic heterogeneous group of diseases, with the largest subgroup showing a mutation in the Nucleophosmin gene (*NPM1*). Normally the NPM protein localizes mainly in the nucleolus, but in AML blasts it is aberrant localized to the cytoplasm (NPMc+AML). Notably, NPMc+AML patients show peculiar gene expression profiles, treatment response and prognosis. Hence, it has been proposed as an independent category for leukaemia classification according to WHO in 2008. In view of the relevance of NPMc+ mutation to AML pathogenesis and prognosis, understanding its role in leukaemia development represents a major issue in the field.

The aim of this PhD project is to get further insight into the relevance of NPMc+ mutations to AML development. To this scope, here it is reported a characterization of a novel mouse model expressing the mutated protein. The hematopoietic restricted expression of the protein induces leukaemia in mice. This data definitively clarify that NPMc+ is an initiating mutation for leukaemia development. However, the long latency and low penetrance of disease onset strongly support the need of cooperating mutations. Since, the high frequency of FLT3-ITD mutations in NPMc+AML, we genetically tested the synergisms between the two abnormalities. To this scope, NPMc+ mice were crossed with FLT3-ITD mice (Lee, 2007). Double mutated mice developed leukaemia with sort latency and full penetrance indicating effective cooperation. Moreover, our data support the two hits model of tumourigenesis, where functional complementary mutations contribute to disease onset.

Another major challenge of this project is to understand how NPMc+ affect the biology of normal HSPC and imposes the transition from normal to cancer stem cells. We found that NPMc+ expression perturbs the homeostasis of HSCP and expand the number of LT-HSC by increasing the proliferation rate. However, this enhanced proliferation is not associated to loss of quiescent and functional HSC, which may represent a reservoir of

persistent pre-malignant cells available for the accumulation of additional genetic alteration. Further investigation into the biology of per-leukaemic stem cells may give insights into the molecular mechanisms imposed by the oncogene for malignancy transformation and finally may contribute for the development of new therapeutic strategies.

4. Introduction

4.1. Nucleophosmin (NPM)

Nucleophosmin (NPM) is an abundant phosphoprotein originally identified as a non-ribosomal nucleolar phosphoprotein that resides within the granular regions of the nucleolus (Kang et al, 1974; Spector et al, 1984). NPM is ubiquitously expressed and highly conserved in vertebrates and it mainly acts as a nucleolar chaperone able to shuttle between nucleus and cytoplasm. Since its identification, several studies have uncovered the complex biology of NPM showing its involvement in different cellular processes such as ribosome biogenesis, genomic stability, response to stress stimuli and regulation of crucial tumour suppressor as ARF (Colombo et al, 2005) and oncogene as c-MYC (Bonetti et al, 2008). Notably, targeting disruption of the *NPM1* gene in mouse showed its essential function during the embryogenesis and, in particular, for the haematopoiesis and brain development (Grisendi et al, 2005).

The interest in NPM has grown following the report published in January 2005 with the contribution of our laboratory of cytoplasmic accumulation of NPM (NPM1c+) associated with mutations in the 3' end of the gene in up to 35% of cases with acute myeloid leukaemia (AML; 60% of patients presenting with normal karyotype). This PhD project constitutes an attempt at further characterization of NPM functions acquired in the presence of the mutation in order to better the understanding of the pathogenesis of AML.

4.1.1. *NPM1* gene and protein structure

The nucleophosmin protein, also known as B23 (Yung et al, 1985), numatrin (Feuerstein & Mond, 1987) or NO38 (Schmidt-Zachmann et al, 1987) is the product of the *NPM1* gene. In the human genome it maps to the chromosome 5q35, spans 25kb and contains 12 exons with sizes raging form 58 to 358 bp. Two alternative splicing isoforms, NPM1 (or B23.1) and NPM1.2 (or B23.2), have been identified form the NPM1 transcript (Wang et al, 1993) (Figure 1). In the long isoform, NPM1 (or B23.1), exon 9 is spliced to exon 11 and the

coding sequence stops at the exon 12, resulting in a protein of 294 residues. The short isoform, NPM1.2, is a truncated protein of 259 residues. For this variant, exon 9 is joined to exon 10 that contains an alternative stop codon, thus the translation stops sooner giving rise to a protein differing from the long isoform in its carboxy-terminal part (Chang & Olson, 1990; Wang et al, 1993). The prevalent form expressed in all tissues is the long isoform that is characterized by abundant expression and the predominant nucleolar localization. The short isoform is expressed at lower level and it is detected mainly in the nucleoplasm. However, its biological significance and physiological function remains largely unknown (Wang et al, 1993). In the human EST database a further transcript variant lacking exon 8 has been identified (GenBank accession number: NM_199185). This variant of 265 residues results in a shorter protein that lacks an internal segment. Its function and its tissue distribution are currently totally uncertain.

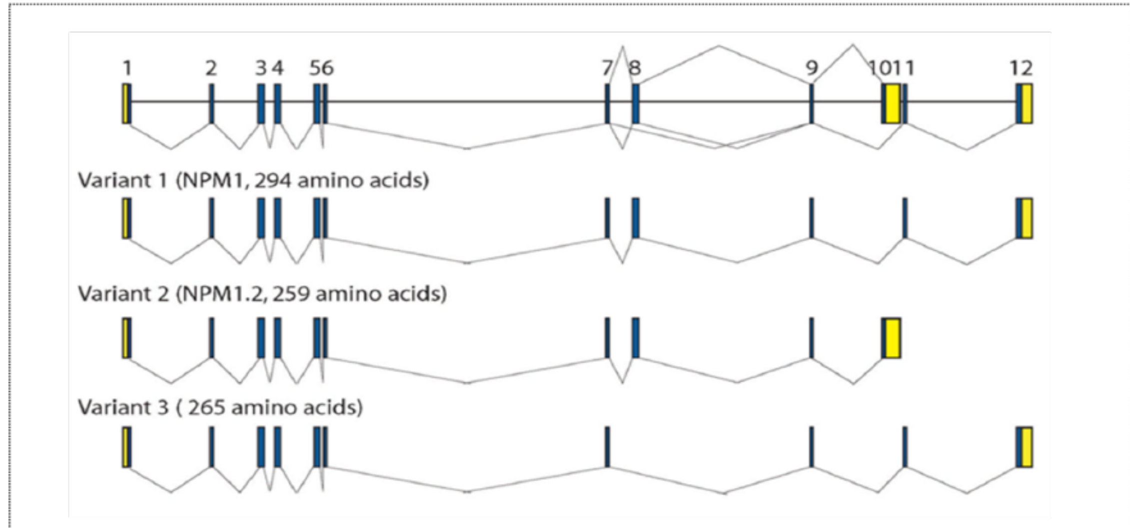


Figure 1 - Schematic representation of the human *NPM1* gene and NPM isoforms variants 1, 2 and 3. Numbers 1-12 represents positions of the exons. The lines in the scheme represent multiple alternative splicing events. Blue and yellow regions represent coding sequence and untranslated regions respectively (modified from Lim and Wang, 2006).

The NPM polypeptide chain has a modular structure containing distinct sequence motifs underlying different functional domains (Figure 2). Starting from the N-terminus, the NPM protein contains a hydrophobic segment that is involved in homo-oligomerization (Herrera et al, 1996) as well as in chaperone activity. Indeed, under native conditions, NPM forms oligomers, preferentially pentamers and decamers (Namboodiri et al, 2004). Sequence analysis has identified in this region a hydrophobic leucine-rich nuclear export signal (NES) motif (LxxPxxLxLx). This non-polar region is followed by two acid stretches that are important for histone binding. The central portion between the two acidic domains is required for ribonuclease activity, together with the C-terminal domain that contains basic regions involved in nucleic-acid binding (Hingorani et al, 2000). The central portion of the protein contains a bipartite nuclear localization signal (NLS) motif. Importantly, in the long isoform alone, the basic cluster is followed by an aromatic stretch, which contains two crucial tryptophan residues (288 and 290) that define a nucleolar localization signal (NoLS) (Nishimura et al, 2002). The presence of both a NES and NLS signals allows for the continuous and rapid shuttling of NPM between the nucleus and the cytoplasm (Borer et al, 1989).

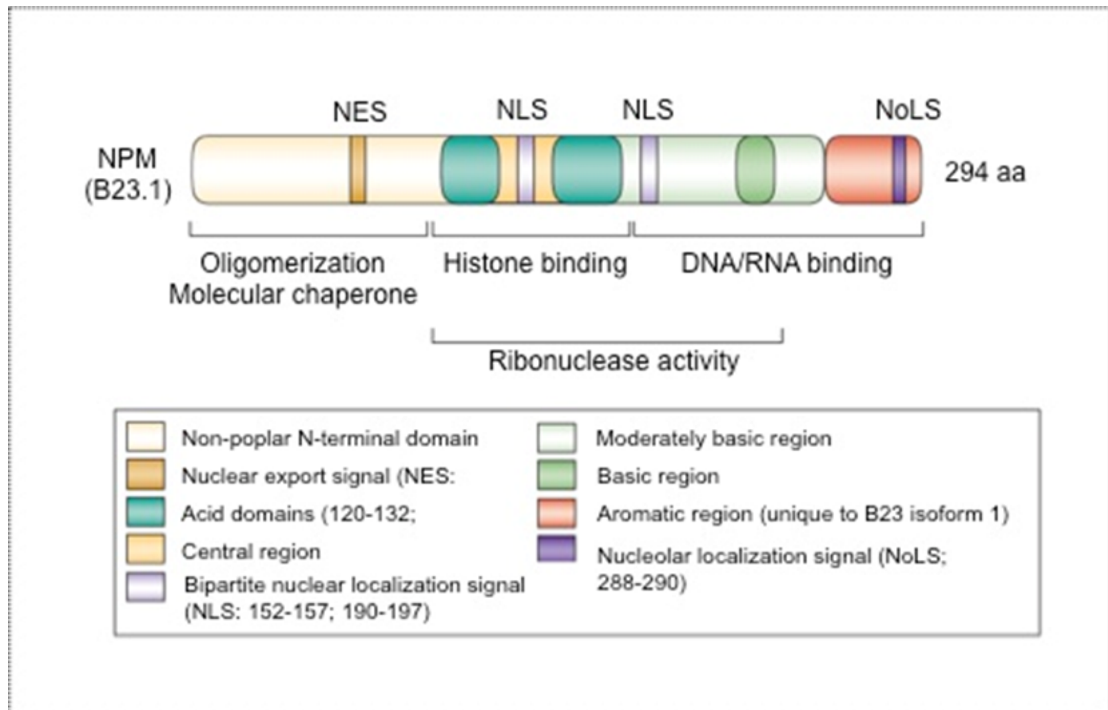


Figure 2 - Schematic representation of the NPM protein.

The different functional domains are indicated and the colour tags are explained in the box. NES: nuclear export signal. NLS: nuclear localization signal NoLS: nucleolar localization signal (modified from Grisendi et al, 2006).

4.1.2. NPM functions

Thanks to different functional domains and its ability to shuttle between the nucleus and the cytoplasm, NPM has been reported to take part in several cellular processes.

4.1.2.1. NPM chaperone activities

Molecular chaperones are proteins that assist the non-covalent folding and assembly among charged molecules within cells, such as nuclear acids or proteins. In this way they prevent aggregation and formation of non-specific complexes.

NPM is a member of the Nucleophosmin/Nucleoplasmin (Np) family of nuclear chaperones, which all share a conserved N-terminal domain (Frehlick et al, 2007). In accordance, several *in vitro* functional studies have reported that NPM behaves like a molecular chaperone on many different substrates (Szebeni & Olson, 1999). Unlike classical molecular chaperone, such as Hsp70 and chaperonins, that release their substrate

using an ATP-dependent mechanisms (Ruddon & Bedows, 1997), *in vitro* experiments showed that the interaction between NPM and its partners is governed by simple association-dissociation thermodynamics, and is not an ATP-dependent process (Szebeni & Olson, 1999). However, it has been observed that NPM is able to bind ATP *in vitro* (Chang et al, 1998). *In vitro* experiments have shown that NPM binds histones and transfers them to relaxed open circular DNA, suggesting a role during the assembly of nucleosome (Okuwaki et al, 2001). Histone chaperones have also been suggested to play a role in removal of histones, during transcriptional activation. In accordance with this, it has been shown that NPM is able to promote nucleosomal disruption (Swaminathan et al, 2005). It seems, therefore, that NPM through its chaperone activity, can influence the transcriptional activity of the cells even if the detailed molecular mechnisms are not compleately understood. Importantly, NPM regulates the half-life and the cellular localization of many of its binding partners, including ARF (Kuo et al., 2004; Colombo et al, 2005) and F-box and WD repeat domain containing 7 (Fbw7γ) (Bonetti et al, 2008) and the SUMO/sentrin/SMT3 specific peptidases 3 and 5 (SENP3-5) (Yun et al, 2008) and influences the stability of the cell cycle inhibitor p21/WAF/CIF (p21) (Xiao et al, 2009).

4.1.2.2. NPM and ribosome biogenesis

Originally, NPM was identified as protein localized in the granular region of nucleolus. For this reason it was believed to be involved in the later stages of ribosome biogenesis (Spector et al, 1984). Additional immunoelectron microscopy studies have revealed that NPM was also present in the dense fibrillar component of the nucleolus (Biggiogera et al, 1989). This region is involved in the processing and maturation of the pre-rRNA transcripts, suggesting a possible involvement of NPM in the initial steps of ribosome biogenesis. It has been shown that NPM, while shuttling between nucleus and cytoplasm, forms specific complexes with other nucleolar proteins such as nucleolin (Li et al, 1996),

p120 protein (Valdez et al, 1994) and HIV-1 Rev protein (Fankhauser et al, 1991). It has been also demonstrated that *in vitro* NPM is able to stimulate the nuclear import of different substrates (Szebeni et al, 1997), containing a Nuclear Localization Signal (NLS). According to these data, it has been proposed a possible role of NPM not only in ribosome assembly but also in the transport of ribosomal and other proteins both inside and outside the nucleolus. Furthermore, *in vitro* studies showed that NPM has an intrinsic ribonuclease activity (Herrera et al, 1996; Herrera et al, 1995). In particular, Savkur and colleagues demonstrated that NPM preferentially cleaves pre-rRNA at a specific site in the internal transcribed spacer number two (ITS2), located 3' to the 5.8S rRNA domain in 32S pre-RNA contributing to the maturation of the rRNA precursors (Savkur & Olson, 1998).

Taken together, NPM is involved at different stages of ribosome biogenesis, maturation of rRNA precursors, and in the trafficking of essential components for ribosome biogenesis. However, it is worth noting that mouse embryonic fibroblasts (MEFs) from mouse lacking the expression of NPM and the tumour suppressor protein p53 are able to grow under culture conditions, thus suggesting that NPM it is not "absolutely required" for ribosomal biogenesis in living cells (Bonetti et al, 2008).

4.1.2.3. NPM and cellular proliferation and survival

NPM is generally considered a protein that promotes cellular growth and survival upon apoptotic/stress stimuli and it appears to regulate these functions in a cell specific manner (Colombo et al, 2011). Indeed, NPM protein level increases in cells that are actively cycling (Dergunova et al, 2002) and in many solid tumour (Grisendi et al, 2006). In fact, NPM has been reported to be overexpressed in colon (Nozawa et al, 1996), gastric (Tanaka et al, 1992), ovarian (Shields et al, 1997) and prostate (Subong et al, 1999) carcinomas, and in some cases the NPM mRNA level correlates with disease progression (Tsui et al, 2004). On the other hand, NPM protein levels decrease when cells are induced to differentiate and the

down-regulation of the NPM mRNA delays mitosis entry (Jiang & Yung, 1999). Furthermore, the proliferation is severely impaired and apoptosis is induced in *NPM1* deficient cells (Colombo 2002) or when NPM nuclear-cytoplasmic shuttling is inhibited (Brady et al, 2004; Grisendi et al, 2005). NPM is involved in proliferation-promoting pathways through different mechanisms. Notably, the *NPM1* gene is a transcriptional target of the c-MYC proto-oncogene and the NPM has been correlated with the stimulation of DNA polymerase-α activity. In addition, several studies have shown that NPM overexpression promotes cell survival in immortal cell lines through the inhibition of specific pro-apoptotic pathways, even though there is no evidence of a direct involvement of NPM in the regulation of the apoptotic machinery. It is likely that NPM acts as an anti-apoptotic protein through indirect mechanisms (Figure 3). In accordance, it has been shown that, in the neuronal PC12 cell line, NPM is required for the anti-apoptotic function of the nerve growth factor (NGF) by inhibiting the DNA fragmentation capacity of the caspase activated DNase (CAD) (Ahn et al, 2005). Another pathway through which NPM exerts an anti-apoptotic response engages the interferon inducible double-stranded RNA-dependent protein kinase (PKR). PKR is an antiviral-response protein that is able to inhibit mRNA translation through the phosphorylation and inactivation of eukaryotic translation initiation factor 2α (EIF2α). Normally, PKR initiates apoptosis in response to different cellular and extra-cellular signals (Jagus et al, 1999). NPM binds PRK and inhibits its pro-apoptotic activities (Pang et al, 2003). Furthermore, it has been demonstrated that although NPM overexpression stabilized the p53 tumour suppressor protein, it prevented the proapoptotic accumulation of p53 in the mitochondria, thus reducing apoptotic response (Dhar & St Clair, 2009). However, another report has shown that in neuronal cells upon apoptotic stimuli, NPM translocates into the cytoplasm where it binds BAX and p53 favouring mitochondrial cytochrome c release and apoptosis (Kerr et al, 2007).

NPM overexpression can also inhibit apoptosis in response to stress stimuli such as UV and hypoxia. NPM overexpression in NIH3T3 fibroblast leads to resistance to UV-induced apoptosis and increase of DNA repair through the inhibition of the IRF1 tumour suppressor, a transcription factor involved in DNA damage-induced apoptosis and cell cycle arrest (Kondo et al, 1997b; Wu & Yung, 2002). Moreover, NPM expression is induced after hypoxia, and human breast tumour cells overexpressing NPM are protected against hypoxic cell death. Indeed, it has been demonstrated that NPM inhibits p53 phosphorylation on serine 15, which is essential for the activation of p53 in response to DNA damage, in response to UV irradiation and hypoxia (Li et al, 2004; Maiguel et al, 2004). Thus, suggesting that hypoxia or UV driven cancer progression may require increased expression of NPM to suppress p53 activation and maintain cell survival.

Although this data suggests that NPM acts as a negative regulator of the p53 tumour suppressor, some other observations have been published, reporting that NPM is involved in p53 stabilization and p53-dependent growth arrest (Colombo et al, 2002; Kurki et al, 2004). Specifically, Colombo and colleagues have demonstrated that NPM interacts directly with p53, both *in vitro* and *in vivo*, and overexpression of NPM in primary diploid fibroblasts induces p53-dependent premature senescence, with concomitant redistribution of p53 to the nucleoli, where it is normally not present. NPM is also able to favour p53 stabilization and activation in response to different kinds of stress and the loss of NPM protein impairs the p53-depedent cellular response to these stresses (Colombo et al, 2002). Similar data have been disclosed by Kurki and colleagues, who showed that depletion of NPM resulted in reduction of both stress-induced and basal levels of p53 (Kurki et al, 2004). Based on these data, NPM could be relevant in the cellular response to radiation and genotoxic stress, through the activation of the p53 pathway.

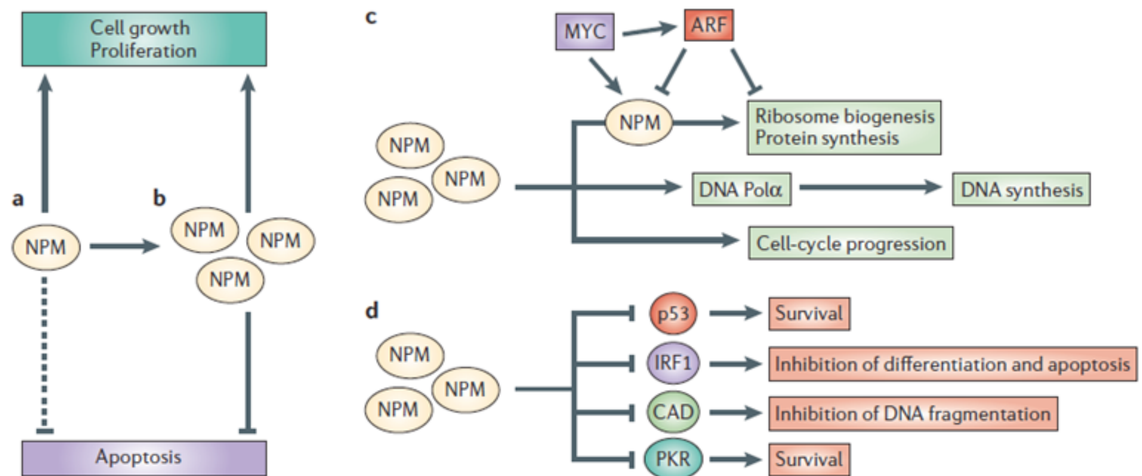


Figure 3 - NPM overexpression in tumour cells leads to increased proliferation and inhibition of apoptosis. a. In normally proliferating tissues, NPM is expressed at physiological levels, and the balance between cell proliferation and apoptosis is maintained. b. In tumour cells, NPM is often overexpressed, and can exert its oncogenic potential both by stimulating cell proliferation and by inhibiting apoptosis. c. NPM overexpression can enhance cell growth and proliferation through the hyperactivation of ribosome biogenesis. NPM is also a target of the c-Myc transcription factor, whose alteration in tumours is often associated with deregulated protein synthesis. In addition, NPM is a putative stimulating factor for DNA synthesis and contributes to cell cycle progression. d. Overexpressed NPM prevents apoptosis through different mechanisms: it prevents the DNA-binding activity of IRF1, which normally induces differentiation and apoptosis, it binds and inhibits the eukaryotic initiation factor 2 kinase (PRK), it counteracts the DNA fragmentation activity of caspase-activated DNAse (CAD), it interacts and inhibits p53 response upon apoptotic stimuli. (From Grisendi et al, 2006).Numbers 1-12 represents positions of the exons. The lines in the scheme represent multiple alternative splicing events. Blue and yellow regions represent coding sequence and untranslated regions respectively (modified from Lim and Wang, 2006).

4.1.2.4. NPM and maintenance of genomic stability

NPM has been widely implicated in the control of genomic stability, both as putative regulator of chromosomal ploidy and as mediator of DNA integrity. Although the specific role for NPM at the centrosome is not fully elucidated, it has been shown that NPM binds centrosome and this association is regulated by different post-transcriptional modifications of the NPM protein during cell cycle (Jiang et al, 2000; Okuda et al, 2000), suggesting a possible role of NPM in controlling chromosome number. Concerning the role of NPM in the maintenance of DNA integrity, it has been shown that, after oncogene activation, NPM

overexpression strongly reduces the level of γ-H2AX, a surrogate marker of DNA damage, and the oncogene-dependent apoptosis or senescence is limited. Importantly, this effect was also confirmed in steady state condition, suggesting that the maintenance of DNA stability is a physiological function for NPM. Indeed, Npm-/- mouse embryos die in uterus at 10.5 dpc, display widespread apoptosis, p53 up-regulation and accumulation of γ-H2AX foci (Colombo et al, 2005). Importantly, these observations were also confirmed in Npm-/- murine hematopoietic precursors derived from the yolk sac, where re-expression of NPM was sufficient to revert this phenotype (Colombo et al, 2005). Moreover, it has been demonstrated that Eμ-Myc transgenic mice show accelerated onset of B-cell lymphoma in an Npm1+/– genetic background. Furthermore, Npm+/– mice are much prone to the development of haematological malignancies late in life with respect to the wild type counterpart, suggesting that NPM acts as haploinsufficient tumour suppressor for haematopoietic disorders (Sportoletti et al, 2008). These data indicate an important role of NPM in the maintenance of DNA integrity although they do not permit to discriminate if the accumulation of DNA damage marked by γ-H2AX phosphorylation is due to direct involvement of NPM in the DNA repair machinery or constitutes an indirect effect of NPM activity on other pathways such as chromatin organization and DNA replication.

4.2. NPM and haematopoietic malignancies

Much of the interest in NPM biology has been bolstered by its involvement in human cancers, and in particular in haematopoietic malignancies. Undeniably, it is one of the most recurrently altered genes. Originally, NPM was studied as a partner of chromosomal translocations in various haematopoietic disorders, including anaplastic large-cell lymphoma (ALCL) with t(2;5) (Morris et al, 1994), the myelodysplasia-AML with t(3;5) (Yoneda-Kato et al, 1996) and cases of acute promyelocytic leukaemia (APL) t(5,17) (Redner et al, 1996). Translocations create chimeric genes encoding for fusion proteins (respectively NPM-ALK, NPM-MLF1, NPM-RARα) that form heterodimers with wild-type NPM and promote cell transformation.

Moreover, the region of the chromosome 5 to which the *NPM1* gene maps is occasionally deleted in some cases of *de novo* myelodysplastic syndrome (MDS) and partial or complete loss of chromosome 5 is observed in therapy related MDS patients (Olney et al, 2002). However, the significance of *NPM1* deletion in MDS remains to be elucidated.

The most striking connection between NPM and haematopoietic malignancies comes from its involvement in acute myeloid leukaemia (AML) pathogenesis.

AML represents a heterogeneous group of diseases characterized largely by bone marrow (BM) failure caused by accumulation of abnormal cells, referred to as blasts. From a morphological point of view, all AMLs are characterized by a blockage of normal haematopoiesis. The grade of blasts differentiation is used to divide the cases of AML in 8 subgroups, M0 to M7, according to the French-American-British (FAB) classification. However, morphological criterion does not incorporate prognostically and therapeutically relevant information. Recent advances in the understanding of the vast genetic and epigenetic variability of AML have provided new insights with regards to the risk stratification of AML patients. In accordance, the newer World Health Organization (WHO)'s AML classification system integrates available genetic information in the attempt

at defining homogenous subgroups of prognostic and therapeutic relevance. The sub-classification and prognostic prediction are first based on cytogenetic abnormalities found in approximately 50% of adult patients with AML. Generally, they are associated with poor or favourable prognosis depending on the underlying translocation (Estey, 2013). The remaining 50% of patients are cytogenetically normal and their prognosis is considered intermediate (Estey, 2013). In recent years, several efforts have allowed to identify mutations that permit further AML grouping, prognostic prediction, and potential development of targeted therapy. Following this challenge, in 2005, Falini and colleagues described mutations in the *NPM1* gene associated to a large subgroup of adult NK-AML (Falini et al, 2005). More in detail, they reported an extensive study of 591 bone-marrow biopsy specimens from patients with primary AML analysed using immunohistochemistry to ascertain the subcellular localization of the NPM protein (Falini et al, 2005). The research samples included patients of different ages (15 to 60 years) and from all of the AML-FAB subtypes. Strikingly, in 35.2% of those specimens, NPM displayed an aberrant cytoplasmic staining (Figure 4), whereas, no NPM delocalization was observed in any of the 135 secondary AML cases analysed or in 980 haematological or extra-hematopoietic neoplasia other that AML (Falini et al, 2005). Due to the peculiar cytoplasmic delocalization of NPM, this subgroup of AML cases was denominated NPMc+AML (c+ indicates cytoplasmic positive) to distinguish from AML where NPM has the expected nucleolus-restricted expression. Finally, the authors demonstrated that mutations in the exon 12 of the *NPM1* gene were predictive of the aberrant cytoplasmic localization of the protein making NPM one of the most frequently mutated genes in AML.

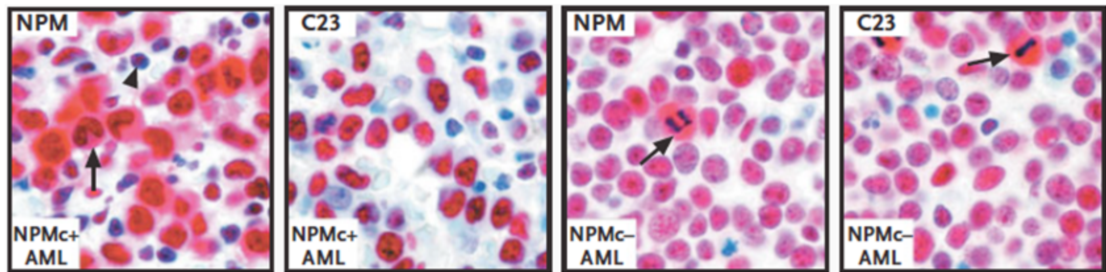


Figure 4 - NPM overexpression in tumour cells leads to increased proliferation and inhibition of apoptosis. Subcellular patterns of expression of NPM in specimens from patients with AML using alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method. In NPMc+AML (two left-hand panels), most leukaemic cells (arrow) show cytoplasmic NPM expression in addition to nuclear NPM expression; the arrowhead indicates a residual hematopoietic cell with the expected pattern of nucleus-restricted NPM. In NPMc+AML, nucleolin (C23) used as a positive control for the nucleolar localization is always restricted to the nucleus. In the two right-hand images, representative of NPMc- AML both NPM and nucleolin (C23) follow the same nucleus-restricted pattern with the exception of mitotic figures that show the expected cytoplasmic expression the two proteins. (Adapted from Falini et al, 2005).

4.2.1. NPMc+ AML defines a distinct disease sub-group

Several lines of evidence suggest that the *NPM1* mutations are a founder genetic lesion in AML (Falini et al, 2011) and represent a distinct group of AML. The cytoplasmic pattern of localization is uniquely associated with *de novo* AML cases (Falini 2005; Liso et al, 2008), in which its frequency is as high as 50-60% of all newly diagnosed cases (Boissel et al, 2005; Falini et al, 2005; Thiede et al, 2006) and it is usually expressed in the whole leukaemic population (Falini et al, 2005). It has been only rarely found in myeloproliferative disorders (Caudill et al, 2006; Oki et al, 2006 ;Ernst et al, 2010) or myelodysplastic syndromes (MDS) (Zhang et al, 2007). Cytogenetic data indicate that NPMc+ staining closely correlates to normal karyotype (NK) (85%). Only a small percentage (15%) of cases bears minor chromosomal abnormalities (Falini et al., 2005) that most likely represent secondary events, since they are present only in a small percentage of blasts (Falini et al, 2005). Importantly, the presence of mutations in the *NPM1* gene is mutually exclusive with other recurrent AML-associated genetic abnormalities such as t(15;17), t(8;21), t(6;9), inv(16) or rearrangements involving 11q23 (Schlenk et al, 2008). Moreover, NPMc+ detection is

stable during the course of the disease in patients (Meloni et al, 2009), likely representing a reliable marker for monitoring minimal residual disease (Schnittger et al, 2009). The hypothesis that NPMc+AML represents a distinct subgroup of AML is reinforced by the existence of specific biological characteristics associated with the disease of these patients. Morphological examination has shown multi-lineage involvement and NPMc+ staining was found in all FAB subgroups with the exception of M3 (acute promyelocytic leukaemia), M4eo (acute myelomonocytic leukaemia with eosinophilia) and M7 (acute megakaryocytic leukaemia) (Falini et al., 2005; Pasqualucci et al, 2006). The multilineage involvement correlated with the negativity of the CD34 antigen (Falini et al, 2005; Taussig et al, 2010) suggesting that NPMc+AML derived from either a common myeloid or an earlier progenitor. However, it has been recently shown that a rare CD34+ population purified from patients' blasts can engraft in immunocompromised mice and support the development of leukaemia that recapitulates the original disease.

Moreover, gene expression profile studies have identified a specific signature, intriguingly, characterized by an up-regulation of homeo-box genes and other genes involved in the stem-cell maintenance (Alcalay et al, 2005).

An additional indication suggesting that NPMc+ AML may represent a distinct group of AML is the peculiar correlation with another frequent genetic abnormality found in AML: FLT3-ITD (internal tandem duplication of fms-related tyrosine kinase 3 gene) (Falini 2005). This mutation is present twice as frequently in cases of NPMc+ AMLs as compared to the cases of NPMc- AML. NPM1 mutations likely precede the appearance of FLT3-ITD, since FLT3-ITD is lost at relapse in approximately 9% of cases (Falini et al, 2011) further suggesting that NPMc+ mutations are a primary genetic event. Interestingly, while NPMc+ AML patients are characterized by good prognosis due to higher remission rate after chemotherapy, the co-presence of FLT3-ITD mutation is associated with poor prognosis (Dohner 2005). In accordance with all these observations, NPMc+AML has

been included as a provisional entity in the 2008 World Health Organization (WHO) classification of lymphoid and hematopoietic neoplasia.

4.2.2. Mutations are located in the exon 12 of the NPM1 gene

From a genetic point of view, mutations in *NPM1* gene are heterogeneous (Falini et al, 2007), with more than 40 mutations reported to date. Beside the molecular variability, *NPM1* mutations share crucial common features. Virtually all of them are restricted to the exon 12 of the gene, with only two exceptions described involving the splicing donor site of NPM1 exon 9 (Mariano et al, 2006) and exon 11 (Albiero et al, 2007). Every mutation generates a shift in the reading frame that alters the C-terminal domain of the protein abrogating at least one of the two tryptophans that are important for nucleolar localization (W288 and W290) (Falini et al, 2006; Mariano et al, 2006) (Figure 5). Notably, NPM mutants not only loose the nucleolar localization signal (WXW), but they also gain a novel leucine-rich nuclear export signal (NES), which is recognised by the CRM1-dependent export machinery responsible for the NPM nuclear export (Yu et al, 2006). The presence of this additional NES is responsible for the cytoplasmic localization of the mutant protein (Mariano et al, 2006). However, despite the variability of mutations described, one mutation, the so called Mutation A, that consists in a duplication of a TCTG tetranucleotide at position 956-959 of the sequence (Gen Bank accession number NM_002520), accounts for about 75-80% of incidence.

Type of Mutation	GenBank Accession No.	Sequence	Predicted Protein
None (wild type)	NM_002520	GATCTCTG....GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLWQWRKSL
Mutation A	AY740634	GATCTCTGTCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation B	AY740635	GATCTCTGCATGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCMAVEEVSLRK
Mutation C	AY740636	GATCTCTGCGTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCVAVEEVSLRK
Mutation D	AY740637	GATCTCTGCCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK
Mutation E	AY740638	GATCTCTG....GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG	-DLWQSLAQVSLRK
Mutation F	AY740639	GATCTCTG....GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG	-DLWQSLEKVSLRK

Figure 5 - Mutations in the exon 12 of the NPM1 gene and in the encoded protein.

The wild-type NPM sequence (nucleotides 952 through 989) is aligned with six mutant variants, called A to F. Red font indicates nucleotide insertion. The predicted protein is always shown, with boxed area indicating the positions of two C-terminal tryptophan (W) residues; the wild type tryptophan residue is shown in yellow, and the mutated residues are in grey. The new amino-acid sequence common to all of the mutated proteins is shown in green. (Adapted from Falini et al, 2005).Numbers 1-12 represents positions of the exons. The lines in the scheme represent multiple alternative splicing events. Blue and yellow regions represent coding sequence and untranslated regions respectively (modified from Lim and Wang, 2006).

4.2.3. NPMc+ and its putative oncogenic pathways

Different molecular mechanisms have been proposed to explain the transforming proprieties of the NPMc+ mutants. Available data suggest that the key event is a domino effect unleashed by the delocalization to the cytoplasm of several NPM interacting partners. An intriguing scenario depicts the mutated protein affecting both a crucial tumour suppressor pathway (Arf-p53) (Colombo et al, 2005) and the stability of a potent cellular oncogene as c-MYC (Bonetti et al, 2008) (Figure 5). Indeed, it has been shown that NPM is required for the nucleolar localization and stability of both ARF and Fbw7γ, a regulator of c-MYC stability (Figure 6).

NPM and ARF. ARF, p19Arf in mouse or p14Arf in human, is a crucial tumour suppressor involved in cell cycle arrest and apoptosis in response to oncogenic stimuli, mainly through the inhibition of MDM2-mediated regulation of p53. MDM2 functions as E3 ligase, which ubiquitinates p53, and is critical for the export of p53 from the nucleus to the cytoplasm, where p53 is degraded (Boyd et al, 2000; Geyer et al, 2000). It was originally proposed that ARF sequesters Mdm2 in the nucleolus where it cannot bind and

ubiquitinate p53 (Weber et al., 1999). NPM associates with ARF in high-molecular weight complexes within the nucleolus (Bertwistle et al, 2004) and stabilizes ARF by retarding its turnover. In leukemic cells, NPMc+ retains the ability to interact with ARF and causes its cytoplasmic delocalization, which in turn affects its normal function. NPMc+ limits the ability of ARF to induce sumoylation of MDM2, and attenuates the ARF-dependent activation of the p53 transcriptional programme (den Besten et al, 2005). Therefore, leukaemia that carry a mutated NPM allele might be characterized by functional inactivation of the ARF tumour-suppressor pathway.

NPM and c-MYC regulation. Fbw7γ is a component of the E3 ligase complex responsible for the proteasome degradation of c-Myc (Yada et al, 2004;(Popov et al, 2007; Yada et al, 2004). In the presence of the NPMc+, Fbw7γ is delocalized into the cytoplasm where it is degraded; as consequence, c-Myc is stabilized (Bonetti et al, 2008). It has been shown that c-Myc overexpression in normal cells promotes an aberrant cellular proliferation leading to the activation of a p53-Arf dependent checkpoint, whose function is to limit proliferation and eventually transformation (Dominguez-Sola et al, 2007). However, in NPMc+ expressing cells the p53/ARF checkpoint is impaired because ARF is delocalized and degraded thus favouring the proliferation and the transformation process (Colombo et al, 2005). Hence, a single genetic mutation in the *NPM* locus may have the double effect of activating proliferation and attenuating the resulting checkpoint response.

However, these data are based on in vitro observations and, consequently, represent an excessive simplification considering the complexity of cellular activities in which NPM is involved and the specificity of the environment in which NPM exert its oncogenic activity. Therefore, an accurate study of the NPMc+ transforming proprieties requires the availability of a mouse model in which NPMc+ is specifically express in the haematopoietic.

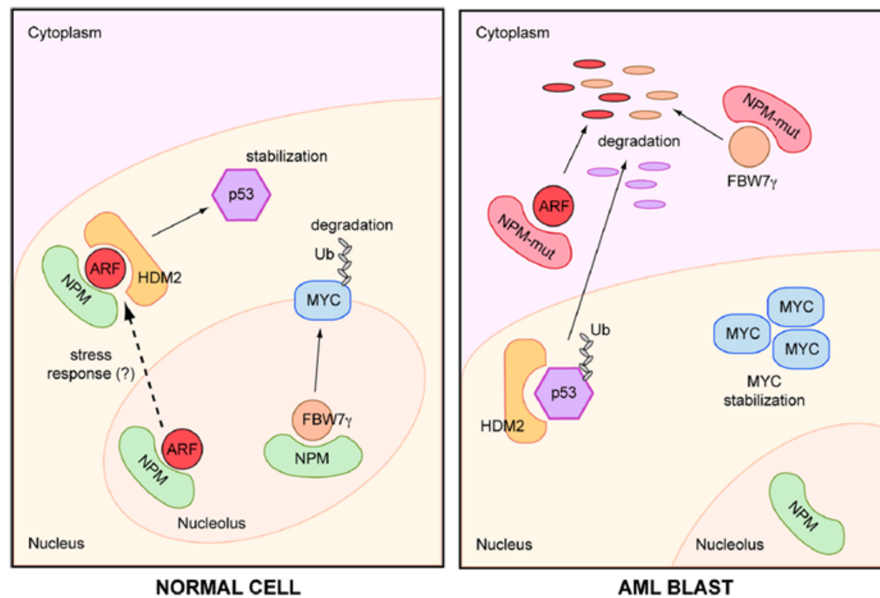


Figure 6 - NPMc+ protein attenuates an oncosuppressor pathway and enhances an oncogenic one. Normal cells: NPM is mainly localized in the nucleolus and is required for nucleolar accumulation and stability of FBW7g and ARF. This is relevant for the control of MYC turnover and provides an active pool of ARF ready to inactivate the HDM2-mediated p53 degradation in response to cellular stress. AML blast: the mutant NPM protein (NPMmut) is mainly localized to the cytoplasm and causes cytoplasmic delocalization and degradation of ARF and FBW7γ. As a consequence, HDM2 can induce ubiquitination/degradation of p53, and MYC accumulates and activates its target genes (from Di Fiore, 2008).

4.3. Acute Myeloid Leukaemia pathogenesis

4.3.1. AML as a multistep process

The fundamental features of AML include inappropriate proliferation, acquired self-renewal potential, escape from programmed cell death, block of differentiation and multi-organ dissemination of leukemic cells. Different lines of evidence support a multistep process AML pathogenesis. First, data from murine models indicate that expression of a single mutated gene is not sufficient to cause AML. For instance, it has been shown that the RUNX1-CBFA2T1 or CBFB-MYH11 fusion genes, resulting from the t(8;21), and inv(16)/t(16;6), respectively, can block myeloid differentiation but are not able to cause frank leukaemia (Castilla et al, 1999; Kelly et al, 2002b; Licht, 2001). Whereas, constitutively activated signalling molecules, such as FLT3 or RAS family member, induce a myeloproliferative phenotype (Kelly et al, 2002; Chan et al, 2004). Furthermore, study in mice directly demonstrated cooperation between different mutations (Kelly et al, 2002a; Schessl et al, 2005; Wang et al, 2011). Moreover, mutational analysis of AML has demonstrated that many patients have more than one recurrent genetic abnormality (Ishikawa et al, 2009). In accordance to these pieces of evidence, the Knudson "two-hit" model of tumourigenesis (Kosmider & Moreau-Gachelin, 2006) proposed that at least two genetic events must take place for a tumour to develop. Thus, it has been suggested that genetic alterations associated with AML can be classified into two complementary functional categories. The class I mutations increase cellular proliferation and survival, typically by constitutive activation of tyrosine kinase signalling pathways, such as mutations in the *FLT3* gene. By contrast, the class II mutations result in a block of normal hematopoietic cell differentiation and/or aberrant self-renewal potential and frequently involve transcriptional regulators of normal hematopoietic differentiation. Murine model bearing class I mutation usually do not develop leukaemia, in contrast to class II mutation. This second group of mutations shows leukaemia initiating potential in mouse, but,

notably, often only following a long period of latency. It has been suggested that during this period a class I mutation is selected and cooperate to give rise to the tumour phenotype. NPMc+ mutations have been proposed to belong to the second class of mutations (Ishikawa et al, 2009).

4.3.2. AML associated mutations

Evidence of the genetic basis of AML initially came from cloning of recurring chromosomal translocations breakpoints associated with malignant phenotypes. Chromosomal translocations are detected in approximately 60-50% of adult AML, and pre-treatment karyotype has long been recognized as the most important independent predictor of clinical outcome (Mrozek et al, 2004). However, 40-50% of AML patients are associated to a normal karyotype and their risk stratification is very heterogeneous (Estey & Dohner, 2006; Lowenberg et al, 2003). The progress in the high throughput sequencing methods has provided the tools for alternative approaches to the identification of disease alleles in NK-AML. Importantly, it has demonstrated the beyond cytogenetic risk classification, molecular genetic markers are clinically significant factors in the response to therapy and survival (Schlenk et al, 2008).). Somatic mutations in AML include partial tandem duplication (PTDs) of the myeloid–lymphoid or mixed-lineage leukaemia gene (*MLL*) (Caligiuri et al, 1994), internal tandem duplications (ITD) (Nakao et al, 1996) or mutations of the tyrosine kinase domain (TKD) (Yamamoto et al, 2001) of the fms-related tyrosine kinase 3 gene *(FLT3*), and mutation in the nucleophosmin gene (*NPM1*) (Falini et al, 2005), the CCAAT/enhancer binding protein α gene (*CEBPA*) (Pabst et al, 2001), and the neuroblastoma RAS viral oncogene homolog gene *(NRAS)* (Bos et al, 1985).

In particular, Flt3 mutations show important association to prognostic factor and will be discussed in more detail as the presence of Flt3 mutation in patients with NPMc+AML drastically worsens the prognosis.

FLT3 mutations. The FMS-like tyrosine kinase 3 (*FLT3*) gene encodes a class III receptor tyrosine kinase (RTK) (Rosnet et al, 1993). FLT3 plays a critical role in normal haematopoiesis (Gilliland & Griffin, 2002) and within the hematopoietic system, its expression occurs primarily in immature myeloid and lymphoid progenitors. Targeted disruption of *FLT3* gene results in healthy adult mice with normal mature hematopoietic populations, however, these animals demonstrate deficiencies in primitive pro-B and pre-B cell lymphoid compartments (Mackarehtschian et al, 1995). These data indicate an important role for FLT3 in the development of multipotent hematopoietic and lymphoid cells. High level of wild-type FLT3 expression has been associated with different haematological malignancies including the vast majority of patients with AML (70-90%) and a large proportion of B cell acute lymphoblastic leukaemia (B-ALL). Different types of mutations affect the *FLT3* gene. Internal tandem duplications (ITD) within the juxtramembrane (JM) domain of FLT3 occur in approximately 25% of AML patients, making it one of the most frequent alterations in adult AML (Frohling et al, 2002). FLT3-ITD mutations result in a ligand-independent receptor dimerization, constitutive FLT3 signalling, and activation of the STAT5, RAS/MAPK, and PIK pathways. Another major class of FLT3 mutations that also cause constitutive FLT3 activation involves the activation loop (AL) of the second kinase domain (Yamamoto et al, 2001) and it is detected in approximately 5%–10% of AML patients. Lastly, a group of point mutations within the JM domain have also recently been described in approximately 1% of AML (Reindl et al, 2006). Gain of function mutation in *FLT3* gene, in particular FLT3-ITD mutations, is of significant clinical consequence. In fact numerous studies have shown that FLT3-ITD mutations may be associated with disease progression, increased risk of relapse and shorter overall survival (Frohling et al, 2002). Furthermore, patients with low or absent levels of wild-type (WT) FLT3, consistent with homozygosity for the *FLT3*-ITD allele, appear to

have a particularly dismal outcome, suggesting *FLT3*-ITD gene dosage has biologic and prognostic significance.

4.4. Haematopoietic stem cells and cancer stem cells

4.4.1. Haematopoietic stem cells (HSC)

Haematopoiesis is the lifelong process by which all cells in the blood are produced in a hierarchical fashion starting from a rare population of multi-potent haematopoietic stem cells (HSC), which are located in bone marrow. Stem cells are cells with a unique fate, as they are clonal precursors of both other stem cells of the same type (self-renewing potential) as well as a defined set of differentiated progeny (differentiating potential) (Weissman, 2000). In accordance, HSCs give rise to progenitor cells that become increasingly lineage restricted and eventually differentiate into all lineages of mature blood cells. However, as HSCs continually replenish cells that turned over, they must self-renew to maintain themselves over the lifetime of the organisms. A complex network of cell intrinsic and extrinsic pathways, whose function is to maintain a homeostatic balance between HSC self-renewal and life-long replenishment of lost blood cells, tightly regulates this process.

So far, HSC are probably the best characterized adult stem cell population with respect to cell surface markers for purification and availability of assays to test functional potential. In 1961, the pioneering work of Till and McCulloch, for the first time, formally demonstrated the existence of rare cells in the mouse bone marrow able to form myeloerythroid colonies in the spleens of irradiated mice transplants (Till & Mc, 1961). These clonogenic population gave rise to same cells that could also be retransplanted to secondary hosts and there reconstitute all blood cell lineages (Becker et al, 1963); (Siminovitch et al, 1963); (Wu et al, 1968). These findings paved the way to the eventual purification of mouse HSC by prospective isolation using fluorescence-activated cell sorting (FACS) with the use of combinations of monoclonal antibody cell surface determinants (Spangrude et al, 1988); (Osawa et al, 1996) or vital dye staining (Goodell et al, 1996). Over the last 20 years, the prospective isolation of HSC has challenged the field with the continual reporting of new

markers for HSC purification improving the purification strategy to yield the highest proportion of HSC (Kiel et al, 2008; Wilson et al, 2008). Therefore, since the discovery of functional HSC, extensive investigations using limiting dilution up to single-cell transplantation assays in combination with the use of unique cell surface markers profile have allowed for the isolation of at least two classes of multi-potent cells with long term (LT-HSCs) and short term (ST-HSCs) reconstitutive potential (Morrison & Weissman, 1994) (Figure 7). The long-term subset self-renews for all the life of the host, while the sort-term subset maintains self-renewal capacity for approximately 8 weeks (Morrison & Weissman, 1994). Understanding the cellular hierarchy of haematopoietic system was then next achieved. At the top of the hierarchy are placed HSCs that self-renew for all the life are placed. These cells progress to multipotent progenitor cells (MPP), an intermediated progenitor stage that commits to one of the two oligolineage-restricted cells: the common lymphocyte progenitors (CLPs) and the common myeloid progenitors (CMP). CLPs are restricted to give rise to T- lymphocytes, B-lymphocytes and natural killer (NK) cells (Kondo et al, 1997a) whereas CMPs are progenitors for the myeloerythroid lineage (Akashi et al, 1999). Then CMP give rise to megakaryocyte-erythrocytes progenitors (MEPs) and granulocyte-macrophage progenitors (GMPs) (Akashi et al, 1999). Finally, these progenitors provide all of the lineage-committed effector cells of the blood system. In normal circumstances, each stage of differentiation of multipotent cells involves functionally irreversible maturation steps and no dedifferentiation or trans-differentiation is permitted between haemato-lymphoid progenitors.

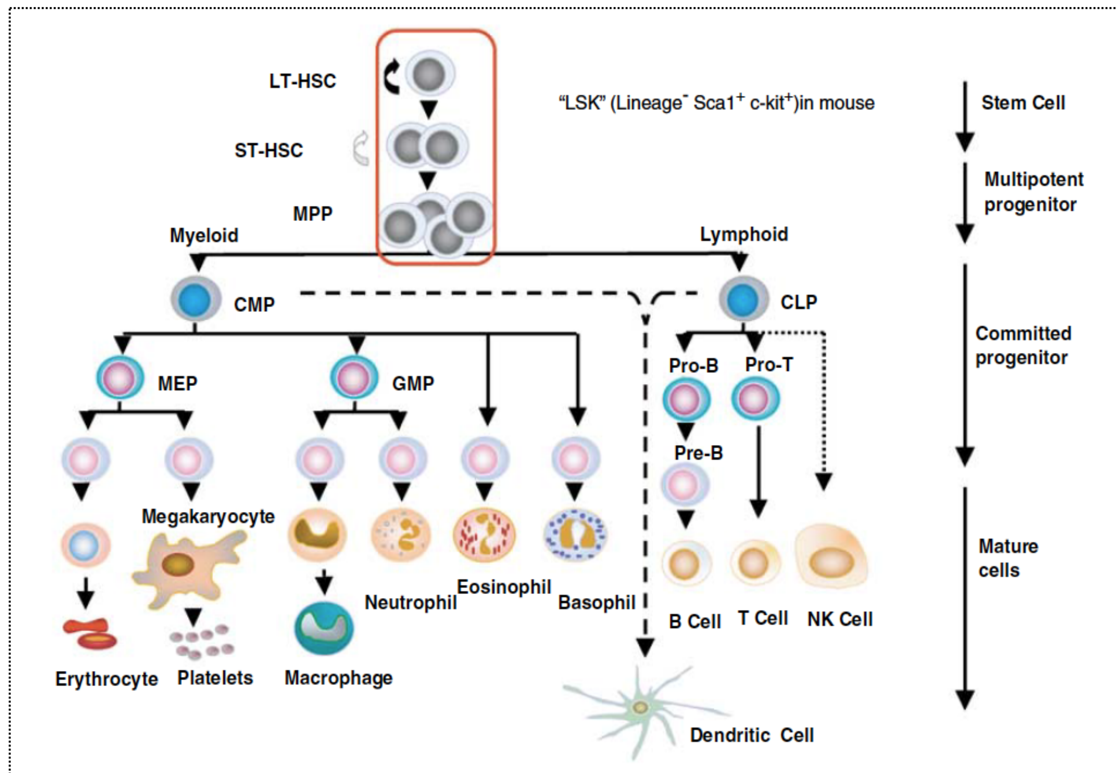


Figure 7 - The hierarchy of the haematopoietic cells.

LT-HSC, long term repopulating HSC; ST-HSC, short-term repopulating HSC; MPP, multipotent progenitor; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte-macrophage progenitor. The encircled pluripotent population, LT-HSC, ST-HSC, and MPP are Lin-, Sca+, c-Kit+, as shown.

4.4.2. Cancer stem cells (CSC)

The classical multi-step model of tumourigenesis requires a long-lived cell in which multiple genetic hits can occur and be propagated (Vogelstein et al, 1988). In accordance, investigators postulated that candidate target cell for tumour initiation could be a stem cell. Similarily to a normal stem cell, the cancer stem cell (CSC) must have self-renewal potential to maintain the tumour, and differentiating/proliferating capability to fuel the tumour bulk (Figure 8). The existence of CSC and hierarchical organization within tumour population is also suggested by clinical data. In many cancers, standard chemotherapy can induce elimination of tumour bulk and disease remission in patients. Unfortunately, a high percentage of those patients will relapse even after a long time (Peloquin et al, 2013; Sorror

& Appelbaum, 2013). This evidence suggests biological heterogeneity within tumour cells (Fidler, 2012). Whereas cells that are efficiently eliminated by therapy compose tumour balk, the cancer-initiating cells, namely CSCs, escape normal chemotherapy based on their different biology (Jordan et al, 2006).

The first formal demonstration of a rare population of cancer-initiating cells came from studies performed on AML patients. In 1994, Dick and colleagues identified a rare subpopulation of cancer cells able to initiate disease in immune-deficient mice (Lapidot et al, 1994). These cells showed differentiating and self-renewing abilities, as they were able to form tumours with the same cell heterogeneity of the tumour of origin, and could be serially transplanted to obtain tertiary tumours. In addition, the surface markers that prospectively identified the AML-initiating cells were found to be specifically expressed in the corresponding normal SCs, as they were CD34+/CD38-. This finding strongly supports the cancer stem cell model, in which a unique rare population, with stem cell characteristics, could support tumour development. Because these AML-initiating cells represented less than the 0.01% of the total leukaemia cells, the authors concluded that rare cells in tumours share the same features as normal SCs and can be referred to as cancer stem cell (CSCs) (Campbell and Polyak, 2007; Visvader and Lindeman, 2008).

Since the first identification in AML, CSCs have been identified in other tumours, including brain and breast cancer (Kai et al, 2010; Singh et al, 2003). Developing new therapeutic strategies that specifically target CSC represents a promising option for the treatment of cancer. However, this goal requires, as an essential prerequisite, a profound understanding of the normal pathways perturbed by oncogene activation, as well as knowledge of the aberrant mechanisms that support the establishment of the clonal dominance during malignant evolution. To this end, the development of proper mouse models that represent a formidable platform to investigate how the transition from a normal to malignant stem cell is necessary.

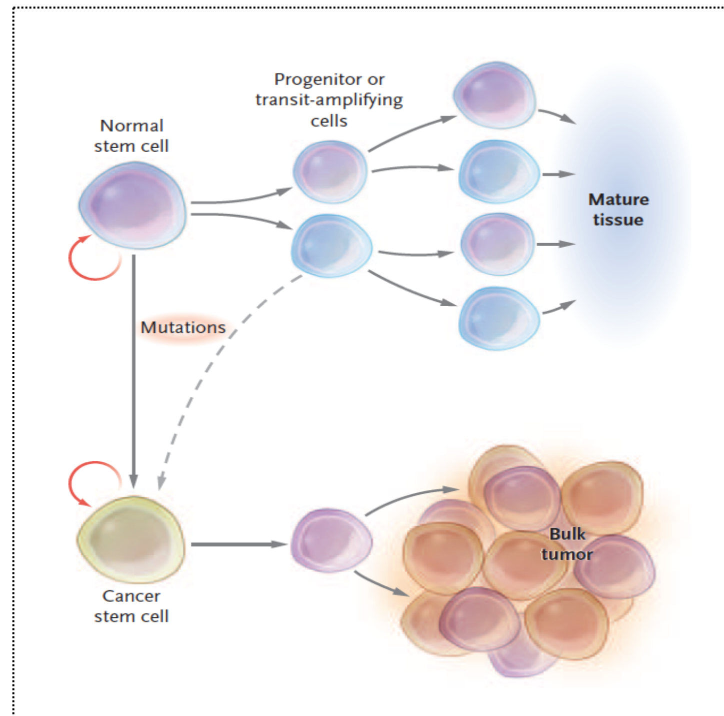


Figure 8 - Stem cell system.

Normal tissues arise from a central stem cell that grows and differentiates to create progenitor and mature cell populations. Key proprieties of normal stem cells are the ability to self-renew (indicated by curved arrow), multilineage-differentiation potential (indicated by cells with different colours) and extensive proliferative capacity. Cancer stem cells arise by means of a mutation in normal stem cells or progenitor cells, and subsequently grow and differentiate to create primary tumours (the broken arrow indicates that specific types of progenitors involved in the generation of cancer stem cells are unclear). Like normal stem cells, cancer stem cells can self-renew, give rise to heterogeneous populations of daughter cells, and proliferate extensively (from Jordan, 2006).

5. Materials and methods

5.1 Animal manipulation

5.1.1 Animal models

NPMc+ mice were described in Results Section, paragraph 6.1.1. FLT3-ITD mice were a kind gift of Prof. Gilliland and were previously described (Lee, 2007). Cre-ER-TM mice and EFYP mice were previously described (Hayashi, 2002) (Srinivas, 2001). C57BL/6 and C57BL/6-Ly5.1 animals were purchased from Charles River.

All animals were kept under specific pathogen free conditions. Mouse experimentations were performed under the protocol number approved by the Italian Ministry of Health.

5.1.2 Histology

Tissue samples were fixed in PBS1X 4% formalin and embedded in paraffin. Haematoxylin and Eosin (H&E) staining were performed according to the following standard protocol.

5 μm sections of bone marrow, spleen, liver, were deparaffinized and hydrated to water, stained with hematoxylin (cat. numb. 03972, Fulka) for 5 minutes, washed in running tap water for 5 minutes, counterstained with eosin (cat. numb. 03972, Fulka) for 3 minutes, dehydrated in 95% and absolute alcohols and mounted.

5.1.3 Cytology

Peripheral blood smear were analyzed using standard May-Grunwald–Giemsa staining protocol. Slide were fixed in methanol 15 minutes, stained in May-Grunwald (MG1L, Sigma) solution for 5 minutes, and then stained in Giemsa (GS500, Sigma) for 10 minutes. At the end samples were rinsed and mounted in histolemon.

5.1.4 Bone marrow transplantation (BMT)

5 million of BM-MNC cells were transplanted in 6.5 Gy irradiated CD45.1 syngenic mice for leukemiogenic study.

For competitive BMT, 1 million of test BM-MNC along with 1 million of competitive BM-MNC were transplanted in 7.5 Gy irradiated CD45.1 syngenic mice.

For serial BMT, 2 million of BM-MNCs were transplanted in 7.5 Gy irradiated CD45.1 syngenic mice.

5.1.5 5-Fluorouracil (5-FU) treatment

5-FU was administrated at dosage of 150 mg/kg by intraperitoneal injection every 7 days until mice death.

5.1.6 5-Bromo-2-deoxyuridine (BrdU) *in vivo* administration

BrdU (BD.pharmngen) was administrated by intraperitoneal injection (1 mg/mouse) at repeated doses every 6 hours.

5.1.7 *In vivo* tamoxifen treatment

Mice were intraperitoneatelly-injected wit 1 mg of tamoxifen (Sigma) 10 times.

5.2 Molecular Biology Techniques

5.2.1 Genomic DNA extraction from tail biopsy

Mouse tissue or cells were lysed o/n at 56°C in 400 ml lysis buffer (100 mM Tris-HCl pH 8; 5 mM EDTA; 200 mM NaCl; 0.2% SDS; 100mg/ml Proteinase K), shaking at 850 rpm, overnight in Thermomixer compact. Undissolved debris were pelleted and DNA in the supernatant was precipitated with 1 ml of isopropanol. The DNA was pelleted by centrifugation, air-dried and resuspended in 400 μl of milliQ water by incubation at 50°C for 20 min.

5.2.2 Genotyping strategy

The genotypes of all mice offsprings were analyzed by polymerase chain reaction (PCR) performed on genomic DNA extracted from tail. For amplification, primers summarized in Table A and PCR reagents in Table B were used. PCR conditions are summarized in Table C/D. PCRs were run in automatic thermocycler GeneAmp PCR System9700 (Applied Biosystems).

5.2.3 Agarose gel electrophoresis and DNA gel extraction

Separation of DNA fragments by size was achieved by electrophoresis in agarose gels (0.7% - 2.5%; 1x TAE (Sambrook & Russell, 2001); 1x GelRed™, BIOTIUM). As a size marker for agarose gel electrophoresis 1kb plus ladder (NEB, USA) was used.

5.2.4 RNA and DNA extraction

Total RNA was extracted from 10^6 cells, using QIAGEN RNeasy kit following the manufacturer's protocol. A DNAse (QIAGEN) digestion step was added before elution to ensure the complete elimination of contaminant DNA.

5.2.5 cDNA synthesis

Complementary DNA (cDNA) was produced using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) following the manufacturer's protocol. Specifically, master mix

(containing 5X VILO™ Reaction Mix, 10X SuperScript® Enzyme Mix, water and RNA) was incubated for 10 min at RT. For increased yields of cDNA incubation time was prolonged to 120 min at 42°C. Reaction was terminated by 5 min incubation at 85°C. 25 ng of cDNA were used for real time PCR reaction.

5.2.6 Real-time quantitative PCR (RT-qPCR)

For gene expression analysis RT-QPCR was performed using 25 ng of cDNA, 500 nM primers mix and 10 µl of LightCycler® 480 SYBR Green I Master, containing Hot-start polymerase (Roche) in a final volume of 20 µl per reaction in 96-well format. Reaction conditions were: 95°C for 10 min, 45 cycles of 95°C for 1 sec, 60°C for 10 sec, 72°C for 1 sec. Accumulation of fluorescent products was monitored using a LightCycler® 480 Real-Time PCR System (Roche). Specificity of PCR reaction was controlled by the melting-temperature profiles of final products (dissociation curve). Ribosomal protein large p0 (Rplp0) gene was used as a control housekeeping gene for normalization (Akamine et al, 2007; Laborda, 1991). Primers are listed in Table E.

Table A: Genotyping primers, annealing temperatures and amplicons

Primers		T_A	Product	
NPMc+			Amplicon	size
5'-TGCTTCAGTCCCATGTTTGGCAAGG-3'	NPMc+ Fw	65°C	Tg	800 bp
5'-AAATCTGTGCGGAGCCGAAATCTGG-3'	NPMc+ Rv			
FLT3-ITD				
5'-AGGTACGAGAGTCAGCTGCAGATG-3'	FLT3_ITD Fw	60°C	Wt	200 bp
5'-GATCATGAACAAAGTCACTGTAAATG-3'	FLT3_ITD rv		Tg	240 bp
eYFP				
5'-GCCATGCCCGAAGGCTACGTCC-3'	YFP Fw	60°C	Tg	280 bp
5'-AGCTGCACGCTGCCGTCCTCGATG-3'	YFP Rv			
CRE-ER-TM				
5'-ACGAACCAAGGTGACAGCAATG-3'	CRE Fw	58°C	Wt	250 bp
5'-CTCGACCAGTTTAGTTACCC-3'	CRE Rv			
HPRT				
5'–TGTGTGTGTGTGTGTGTAGGTCACC-3'	HPRT Fw	60°C	Wt	800bp
5'-GGTTGCTGGAGTGCTTGCC-3'	HPRT Rv			

Table B: Master mix used for PCR genotype

PCR master mix	µl
H2O	15.3
10x CoralLoad PCR buffer (contains 15mM MgCl2)	2.5
5x Q solution	5
dNTPs 10 mM	0.5
primer Fw 100 µM	0.25
primer Rv 100 µM	0.25
Taq DNA Polymerase	0.2
DNA	1
Total	25

Table C: PCR condition for genotype (CRE-ER-TM, FLT3-ITD, HPRT, YFP)

PCR cycle	
Temperature (°C)	Time (min)
95	5
94	0:30
T_A	0:30
72	0:30
72	7:00
4	Hold

X 35

Table D: Condition used for PCR genotype (NPMc+)

PCR cycle	
Temperature (°C)	Time (min)
95	5:00
94	0:30
65	0:30
68	5:00
72	8:00
4	Hold

X 35

Table E: Primer sequences for quantitative PCR analyses

Primers	Sequence	Target
HOXA5 Fw	CGCAAGCTGCACATTAGTCA	RNA
HOXA5 Rv	CATCCTCCTGTTTTGGAACC	RNA
HOXA7 Fw	GCCTCCTACGACCAAAACAT	RNA
HOXA7 Rw	CTTCTCCAGTTCCAGCGTCT	RNA
HOXA9 Fw	ATGCTTGTGGTTCTCCTCCA	RNA
HOXA9 Rw	CTTCTCCAGTTCCAGCGTCT	RNA
HOXA10 Fw	CAGCCCCTTCAGAAAACAGT	RNA
HOXA10 Rw	CGCTACGGCTGATCTCTAGG	RNA
HOXB6 Fw	CCGCATAGCCAGACGAGTAGA	RNA
HOXB6 Rw	TTCCTATTTCGTGAACTCCACCTT	RNA
TATA box Protein (TBP) Fw	CTGGAATTGTACCGCAGCTT	RNA
TATA box Protein (TBP) Rw	TCCTGTGCACACCATTTTTC	RNA

5.3 Cell Culture

5.3.1 Preparation of BM-MNCs and spleen cell suspension

Single-cell suspensions were obtained by grinding bone marrow or spleen by crashing followed by filtering through 70 mm nylon meshes (Becton Dickinson, USA).

BM-MNCs were layered onto density gradient (HISTOPAQUE 1083, SIGMA – ALDRICH) upon centrifugation at 14C at 1500 rpm for 35 minutes. During centrifugation, erythrocytes are aggregated by polysucrose and rapidly sediment; whereas, lymphocytes and other mononuclear cells remain at the plasma-HISTOPAQUE interface.

Erythrocytes lyses from peripheral blood was achieved incubating cell suspensions in 1 ml of a 9:1 (v/v) solution of 0.15M NH_4Cl and 0.17M Tris-HCl pH 7.65 for 3 min on ice. Cells were centrifuged at 1200 rounds per minute (rpm) at 4°C and.

Live cells were counted using Trypan blue dye to distinguish live and dead cells.

5.3.2 Colony-forming unit (CFU) assay

Myeloid colony-plating assays were performed in methylcellulose- based medium (M3434) containing 3 U/ml erythropoietin, 10 ng/ml recombinant murine interleukin-3 (rmIL-3), 10 ng/ml rmIL-6, and 50 ng/ml rmSCF (Stem Cell Technologies, Vancouver, BC, Canada). For primary methylcellulose cultures, cells were seeded in triplicate and scored for colony formation 7–10 days later. Serial replating was carried out as previously described (Lee, 2007) in three independent experiments with 10^4 cells replated in duplicate for each round of replating and colony counts performed on 7-10 days.

5.3.3 TAT-Cre transduction of BM-MNCs

BM-MNCs were washed three times, re-suspended in serum-free media (Hyclone, USA) at a density of $5x10^6$ cells/ml and incubated for 45 min at 37°C with 100 μg/ml (final concentration) of recombinant TAT-Cre. Transduction was stopped diluting samples with 10 volumes of BM-MNCs medium (IMDM (Gibco/Invitrogen, Carlsbad, CA) plus 12.5% heat inactivated fetal bovine serum (FBS), 12.5% Horse serum, 1% L-glutamine,

100ng/mL SCF, 20ng/mL IL3, and 20ng/mL IL6 (Pepro Tech, Rocky Hill, NJ), 0.1% β-mercaptoethanol, and Hydrocortisone 10ng/mL.), cells were spun down, re-suspended in BM-MNCs medium and cultured at a density of 2 x 10^6 cells/ml. Deletion efficiency was evaluated 24-48 hours later by flow cytometry.

5.4 Imaging analysis

5.4.1 Flow cytometry and cell sorting analysis

For flow cytometric analysis aliquots of $1x10^6$ cells were stained for 30 minutes on ice in 100 μl FACS buffer (1% PBS, 1% BSA, 0.01% Sodium Azide (N_3) containing the appropriate mixture of fluorescently labeled antibodies (Abs). Two-step staining was performed when biotinylated Abs were detected using fluorescently-labeled streptavidin as secondary reagent. Stained cells resuspendend in FACS buffer were acquired on a FACSCalibur (BD Pharmingen, USA) and data were analyzed using Flowjo software (Tree Star, USA). Dead cells were excluded from the analysis based on forward and side scatter parameters. Sorting of labeled cells was performed with a FACS Vantage or FACS Aria (BD Pharmingen, USA). Monoclonal Abs were purchased from eBioscience.

List of Abs as well as working dilutions are listed in Table F. Lineage negative cell (Lin-cell) were identified according to the low expression of MAC1; Gr1; B220; CD3; Ter119.

LT-HSC were identified according to the following immunophenotype (Lin-; SCA+; cKit+; CD34-; FLK-). ST-HSC were identified according to the following immunophenotype (Lin-; SCA+; cKit+; CD34+; FLK-). MPP were identified according to the following immunophenotype (Lin-; SCA+; cKit+; CD34+; FLK+).

Table F: List of antibodies used for flow cytometry

Antibodies and antigen (clone)	Source	Dilution factor
Monoclonal Rat–anti rat mac (clone M1/70)	eBiosciences	1/200
Monoclonal Rat–anti rat GR1 (clone RB6-8C5)	Home-made	1/200
Monoclonal Rat–anti ratB220 (clone RA3-6B2)	Home-made	1/200
Monoclonal Rat–anti rat CD3 (clone 145-2C11)	eBiosciences	1/200
Monoclonal Rat–anti rat ter119 (clone TER-119)	eBiosciences	1/200
Monoclonal Rat–anti rat Sca-1 (clone E13-161.7)	eBiosciences	1/100
Monoclonal Rat–anti rat ckit (clone 2B8)	eBiosciences	1/100
Monoclonal Rat–anti rat CD34 (clone RAM34)	eBiosciences	1/100
Monoclonal Rat–anti rat FLK (clone A2F10)	eBiosciences	1/100

5.4.2 Cell cycle analysis

Surface stained cells were fixed in BD Cytofix/Cytoperm™ buffer for 20 min at room temperature (RT), washed by Perm/Wash™ Buffer (P/W) and re-fixed in BD Cytofix/Cytoperm™ Plus buffer for 10 minutes, at RT, light protected. Cells were washed with P/W and incubated 5 minutes with BD Cytofix/Cytoperm™ buffer, at RT, light protected, and washed again with P/W. Fixed cells were treated with 300 mg/ml DNAse for at least 1 hour, at 37°C, washed with P/W, and stained with anti-BrdU-APC (BD Pharmingen, Cat: 51-23619L) conjugated antibody, 30 minutes, RT. Stained cells were washed two times, re-suspended in 1 ml of cold PBS containing hoacst (50 μg/ml, Sigma), and incubated overnight at 4°C. Samples were acquired by FACS-ARIA (BD) and analyzed using FlowJo software.

5.4.3 Immunofluorescence

Cells were fixed in suspension with 4% paraformaldehyde for 10 minutes, washed three times in PBS1X and plated onto polilysinated coverslips. Cells were then permeabilized 5 minutes with 0.1% Triton-X100 in PBS at room temperature, washed three times in PBS and blocked with 3% BSA in PBS (blocking solution) for 20 minutes. Staining with primary antibodies was performed in a humid chamber for 1 hour at room temperature and followed by three washes in PBS1X. Coverslips were then stained with secondary antibodies for 30 minutes at room temperature, washed three times in PBS1X, counterstained with DAPI and mounted in mowiol. Samples were analysed under an AX-70 Provis (Olympus) fluorescence microscope equipped with a b/w cooled CCD camera (Hamamatsu c5985). In this work a mouse monoclonal NPMc+ antibody (T26) (1:800) or a mouse monoclonal NPM (322) (Falini, 2005) was used followed by an anti-mouse Cy5 antibody (1:100 in blocking solution, Jackson Laboratories).

5.5 Biochemical techniques

5.5.1 Immunoblotting analysis

Cells were harvested by centrifugation for 5 min at 2400 rpm and washed in cold PBS1X. For protein extraction cell pellet was resuspended in 8 M Urea lysis buffer (8 M Urea, 0.1 M NaH_2PO_4, 0.01 M Tris pH 8.0) or RIPA buffer (10 mM Tris-HCl, pH 8.0, 1% Triton, 0.1% SDS, 0.1% Deoxycholate, 140 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and protease inhibitor cocktail Set III (EDTA-free, Calbiochem,), and incubated 1 hour on ice. Sonication was performed in 2-3 cycles of 10 sec each. Lysates were centrifuged at maximum speed for 10 min to remove debris. Lysates were quantified with a protein assay reagent (Bio-Rad Laboratories). 20 - 50 μg of proteins, in Laemmli loading buffer (62.5 mM Tris-HCl pH 6.8, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 10% (v/v) glycerol) and 10 μl of NOVEX ® Sharp Pre Stained protein standard (Invitrogen) were run onto 10% SDS-polyacrylamide gel electrophoresis and transferred by dry iBlot® Dry Blotting Device (Invitrogen) to nitrocellulose membranes (iBlot® Transfer Stack, Invitrogen) in 6 minutes. After blotting, the membranes were stained with Ponceau S staining solution (0.1 % Ponceau S (w/v) and 5% acetic acid (w/v) to verify equal loading and transfer. Membranes were briefly washed in water and blocked for 1 h at RT in blocking solution 5% BSA, or 5% milk, in TBS-T (20 mM Tris HCl pH 7.4, 500 mM NaCl, 0,1 % Tween). After blocking, filters were incubated with primary antibodies, diluted in blocking solution, for 1-2 hours at RT or overnight at 4°C. After three washes, of 5 min each in TBS-T, membranes were incubated with the appropriate horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking solution for 30 min, at room temperature. Membranes were washed three times for 5 min in TBS-T and the bound secondary antibody was revealed using the ECL (Enhanced Chemiluminescence) plus method (Amersham).

5.6 Statistical analysis

5.6.1 Student's t-test

Statistical analysis of normally distributed values (Gaussian) was performed by two-tailed unpaired Student's t-test. Differences were considered significant at *p-value*<0.05.

5.6.2 Survival analysis

Survival curve was analyzed with log rank test.

6. RESULTS

6.1 NPMc+ initiates leukaemia in a novel mouse model

6.1.1 Generation of conditional mouse model for the expression of human NPM1c+ mutation

To better understand the role of the cytoplasmic mutations of the *NPM1* gene in disease pathogenesis, a novel conditional *NPM1c+* mouse model was generated in our laboratory in collaboration with GenOway (Leon, France). The human *NPM1* mutation A cDNA has been inserted in the permissive hypoxanthine phosphoribosyltransferase (*hprt*) locus via homologous recombination in embryonic stem (ES) cells (Figure 9 A). The project was developed in 129Ola E14 ES cells in which 35 kb of the *hprt* gene, encompassing the 5' UTR up to intron 2, is deleted. The homologous recombination between the targeting vector and the *hprt* locus repaired the *hprt* gene deletion and Hypoxanthine-Aminopterine-Thymidine (HAT) medium was used to enrich for ES cell clones with correct targeting event (Doetschman, 1987). The targeting vector included two homologous regions to the *hprt* locus, a STOP cassette flanked by two loxP sites that can be removed upon Cre recombinase induction, thus allowing for the expression of the *NPM1* mutant under the control of the ubiquitous and strong CAG promoter (Alexopoulou et al, 2008) (Figure 9 A). ES cells that have undergone homologous recombination, as determined by Southern blot analysis (Figure 9 B-C), were microinjected into C57BL/6 blastocysts to generate chimeric mice. Following germ-line transmission from chimeric mice, the resulting heterozygous progeny (NPMc+/WT) was intercrossed and analysed by Southern blot to confirm correct targeting of the transgene at *hprt* locus. Animals were backcrossed in C57BL/6 strain 10 times to obtain a pure strain. Considering the location of the *hprt* locus on the X chromosome, the present study was developed using hemizygous NPMc+/WT males or homozygous NPMc+/NPMc+ females, hereafter, these mice are collectively referred to as NPMc+ mice. All the animals carrying the NPMc+ transgene born at Mendelian ratio, they were viable and fertile, and they did not manifest any gross phenotypic abnormalities.

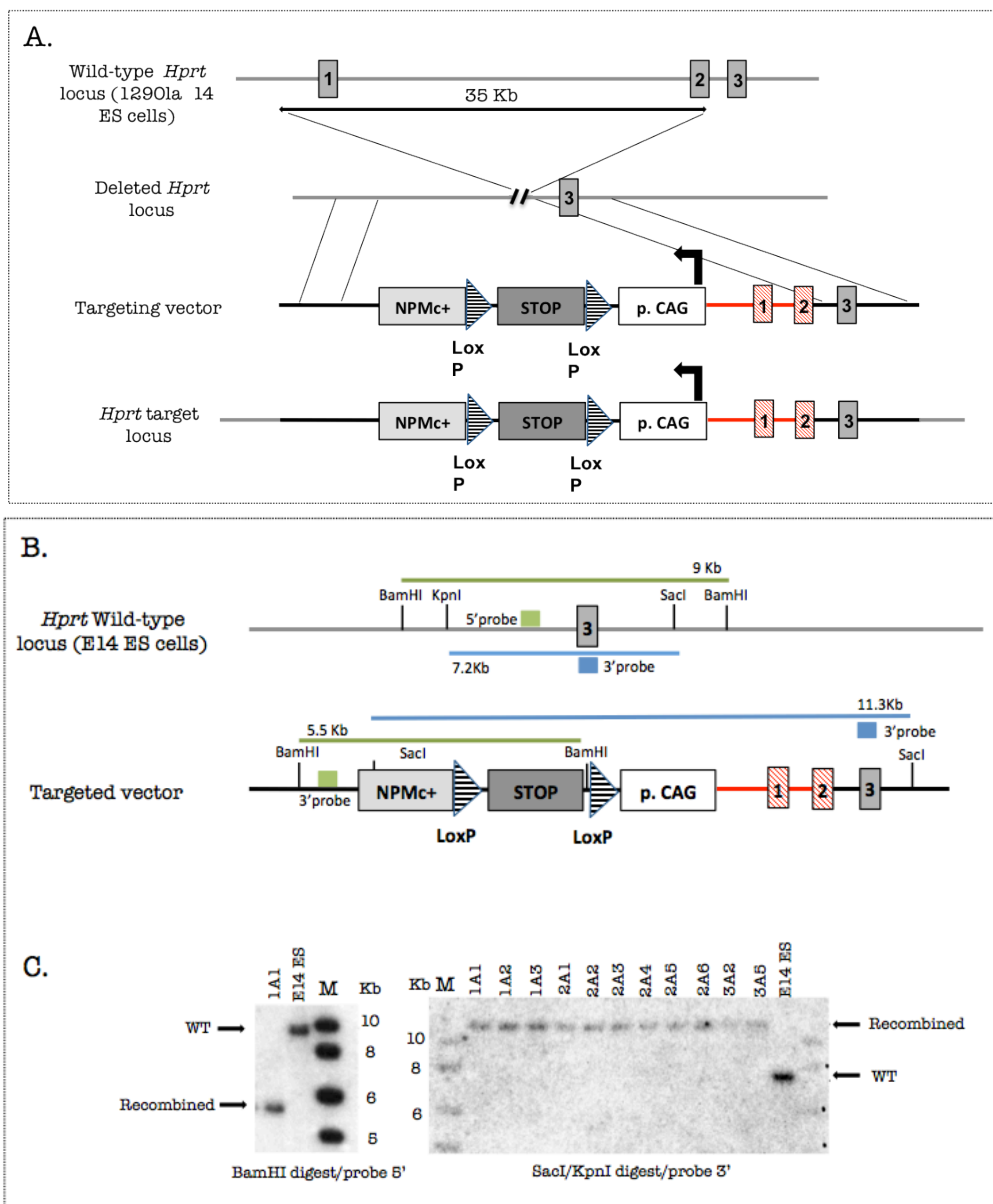


Figure 9 - Generation of a conditional mouse model with the human NPM1 mutation.

A. Strategy for targeted insertion of the human NPM1 mutation A cDNA into the murine *hprt* locus. The picture depicts the structure of the mouse *hprt* genomic locus in wild type (WT) mice (on the top), in *hprt* partially deleted E14 ES cells (upper middle), the targeting vector (lower middle) and the target allele (bottom). Diagram is not drawn to scale. B. Schematic representation of the *hprt* locus in E14 ES cells (on the top) and the homologous targeted allele (on the bottom) with the relevant restriction sites for the Southern blot analysis. C. Southern blot analysis confirmed homologous recombination. (Image adapted from GenOway).

6.1.2 Conditional expression of NPM1 mutation in the haematopoietic compartment

In order to restrict the expression of the NPMc+ protein to the haematopoietic compartment and avoid unknown effects linked to the expression of the mutated protein in other tissues, bone marrow mononuclear cells (BM-MNC) from NPMc+ (8 to 10 weeks old) or age matched control wild type (WT) mice were purified, treated *ex vivo* with a recombinant Cre protein fused with the HIV-derived TAT protein (TAT-Cre) and then transplanted in C57BL/6 CD45.1 recipient irradiated mice (Figure 10). The recombinant TAT-Cre protein is able to penetrate into the cells and to target floxed sequences (Peitz et al, 2002) thus allowing the expression of the NPMc+ protein.

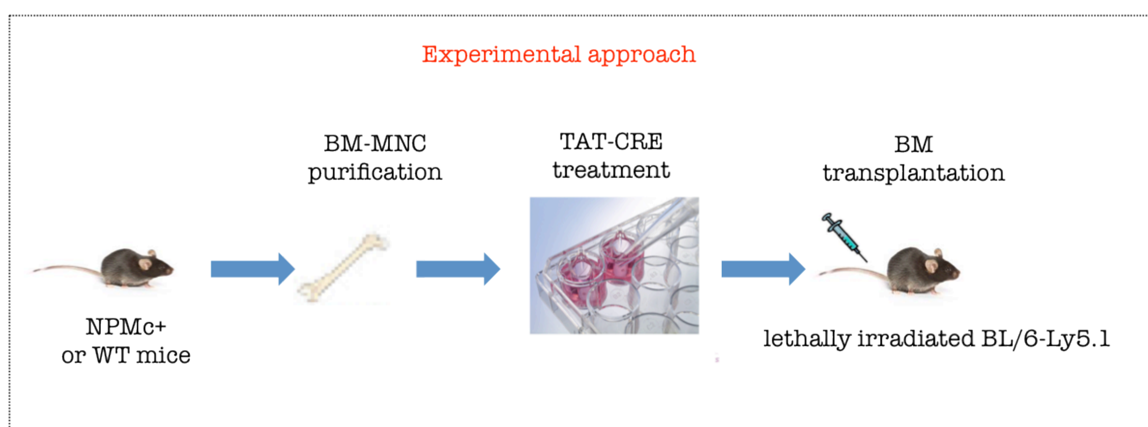


Figure 10 - Schematic representation showing experimental design to induce *NPM1c+* expression in the haematopoietic compartment.
BM-MNCs were isolated from WT or NPMc+ mice and treated ex vivo with TAT-Cre to induce recombination and deletion of the STOP cassette. TAT-Cre treated cells have been transplanted into 6.5 Gy irradiated C57BL6/ CD45.1 mice.

The efficiency of TAT-Cre mediated recombination and successful expression of the NPM mutant protein was assessed both by western blot and immunofluorescence using an antibody that specifically recognizes only the NPM mutant protein (T26) (Gruszka et al, 2010). Western blot analysis clearly revealed the expression of the NPMc+ protein upon TAT-Cre treatment (Figure 11A) and the percentage of recombined cells was evaluated by

immunofluorescence (38.9% +/- 5.15 in all experiments). As previously reported (Falini et al, 2005), the mutated protein clearly accumulated in the cytoplasm (Figure 11B). The delocalization of the mutated protein was further confirmed using an antibody that recognizes both NPM WT and mutated (322) (Falini et al, 2005). In this case, NPM displayed nucleolar/nuclear staining in control cells and both nuclear/cytoplasmic staining in NPMc+ expressing cells (Figure 11C).

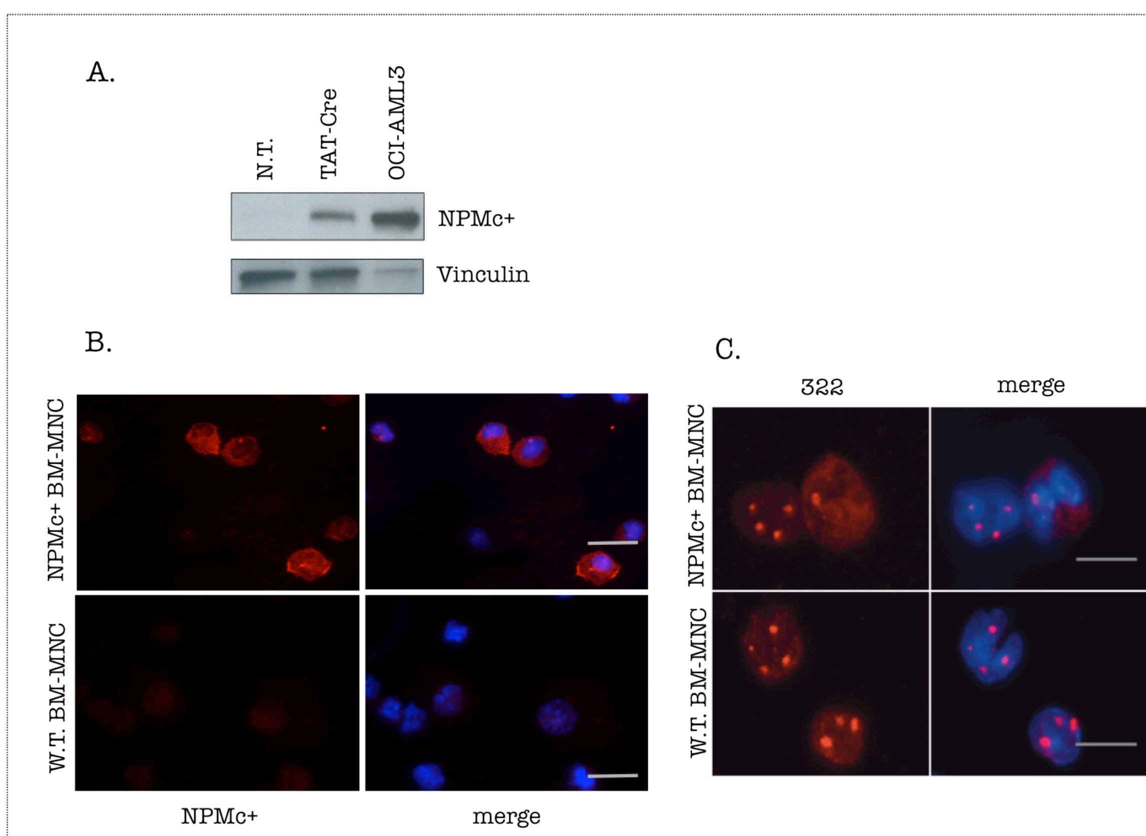


Figure 11 - Expression of the NPM1 mutation in TAT-Cre treated NPMc+ BM-MNCs.

A. Western Blot analysis of NPMc+ protein expression with mAb specific to NPM1mut A protein (T26) in TAT-Cre treated NPMc+ BM-MNCs. OCI-AML3, a NPMc+ expressing cell line, was used as positive control; NT, not treated NPMc+ BM-MNCs. B. Immunofluorescence analysis of TAT-Cre treated NPMc+ BM-MNCs labelled with the same mAb used in (A) (red staining). NPMc+ cytoplasmic staining is highlighted merging the red staining with DAPI nuclei staining (merge). C. Immunofluorescence analysis of TAT-Cre treated NPMc+ or WT BM-MNCs labelled with an antibody that recognizes both the WT and the mutant NPM (red staining) (clone 322). Original magnification 60X. Scale bar = 10 μm.

In order to evaluate the extent of engraftment, 4 weeks after bone marrow transplantation (BMT) mice were tested for donor haematopoietic reconstitution using the CD45.1 and CD45.2 allelic variant discrimination system (Figure 12A). As shown in figure 12B, the average reconstitution in mice was 84,45%±3,8 for NPMc+ animals and 85,02%±4,67 for WT animals, remarkably, the reconstitution was maintained during 24 months of follow up (Figure 12C). Moreover, NPMc+ protein expression was detected in the peripheral blood of transplanted mice 4 months after bone marrow transplantation (Figure 12D), indicating effective long-term reconstitution supported by NPMc+ expressing haematopoietic stem cells (HSC).

Altogether these data indicate that we have generated a novel mouse model expressing the cytoplasmic delocalized NPMc+ mutation whose expression is restricted to the haematopoietic compartment. This model represents a useful tool to study the impact of NPMc+ mutation on both haematopoiesis and leukaemogenesis.

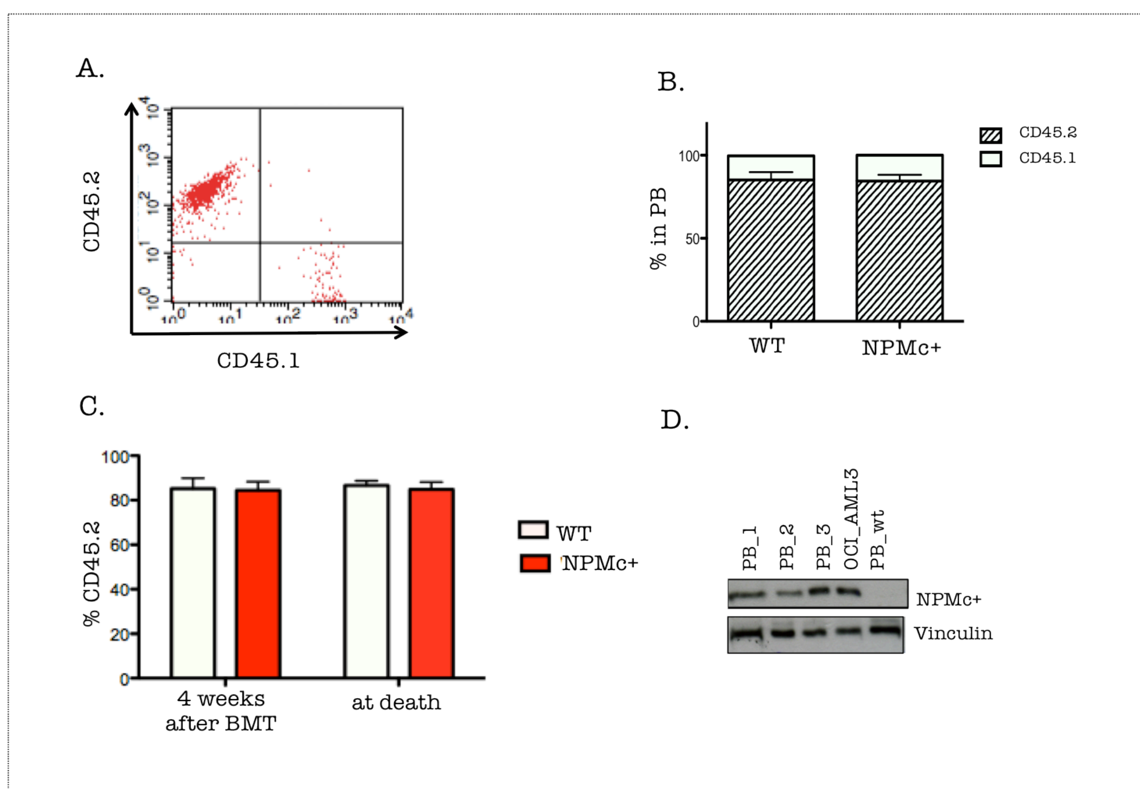


Figure 12 - Haematopoietic reconstitution of NPMc+ and control BMT mice.

A. Representative FACS plot for gating strategy. Peripheral blood was analysed for the relative contribution of donor (CD45.2) and recipient (CD45.1) mice. B. Summary chart representing CD45.2 and CD45.1 chimerism in peripheral blood (PB) of WT or NPMc+ BMT mice at 4 weeks after BMT. C. Summary representing the CD45.1 and CD45.2 chimerism in peripheral blood of WT or NPMc+ BMT mice 4 weeks after BMT and at time of death. (WT N=17; NPMc+N=15) D. Western blood of BMT mice 4 months post-BMT. (PB_1,2,3 represent blood from three NPMc+ BMT mice; PB_wt represents blood from a control WT BMT mouse; OCI_AML3 is used as positive control for NPMc+ expression.A. Western Blot analysis of NPMc+ protein expression with mAb specific to NPM1mut A protein (T26) in TAT-Cre treated NPMc+ BM-MNCs. OCI-AML3, a NPMc+ expressing cell line, was used as positive

6.1.3 NPM1c+ mice develop leukaemia late in life

To study the ability of NPMc+ mutation to promote leukaemia development in our model, NPMc+ and WT BMT mice were allowed to age. Mice were monitored three times at week for general health conditions by global examination and palpation. Every month peripheral blood was collected and detailed haematological analysis was performed by differential blood cells count and blood smear inspection. Animals were sacrificed at first signs of pain and organs and cells were collected for detailed studies, as described below. Overall survival of the two groups of animals was not significantly different with a median survival of 581 and 694 days in NPMc+ and control mice, respectively (Figure 13A). Strikingly, 33.3% of NPMc+ animals developed leukaemia late in life compared to no leukaemia derived death in control mice (p=0.04) (Figure 13B), while the incidence of diseases other than leukaemia did not differ between the two groups (Figure 13C and Table 1). Interestingly, two NPMc+ animals died of severe anaemia with myelodysplastic features in the bone marrow and peripheral blood smear.

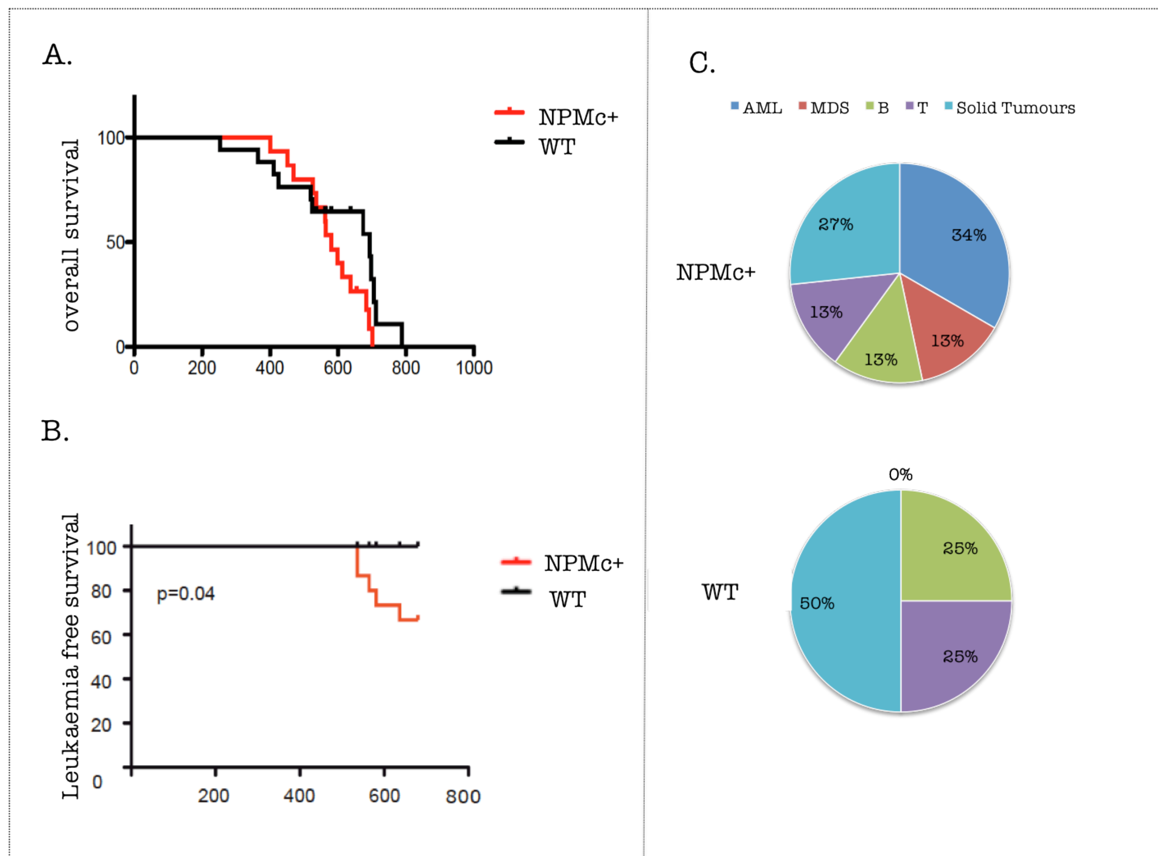


Figure 13 - The NPMc+ mutation promotes leukaemia development in NPMc+ BMT mice.

A. Kaplan-Mayer survival curve of NPMc+ and WT BMT mice showing a comparable overall survival between the two groups. B. Kaplan-Mayer curve showing statistically significant mortality due to AML in NPMc+ cohort compared to WT mice. C. Pie chart representation of disease incidence in NPMc+ or WT mice. AML=Acute myeloid leukaemia; MDS, myelodysplastic syndrome; B, T= B or T lymphoma. N=17 WT and N=15 NPMc+ BMT animals.

Table 1. Diagnostic findings for NPMc+ and WT transplanted mice.

Mouse ID	Genotype	Putative cause of death
N_1	NPMc+	AML
N_2	NPMc+	AML
N_3	NPMc+	AML
N_4	NPMc+	AML
N_5	NPMc+	AML
N_6	NPMc+	Severe anaemia (MDS)
N_7	NPMc+	Severe anaemia (MDS)
N_8	NPMc+	B-lymphoma
N_9	NPMc+	B-lymphoma
N_10	NPMc+	T-lymphoma
N_11	NPMc+	T-lymphoma
N_12	NPMc+	Stomach cancer
N_13	NPMc+	Retrobulbar tumour
N_14	NPMc+	Retrobulbar tumour
N_15	NPMc+	Not exanimated
W_1	WT	Sacrificed
W_2	WT	Sacrificed
W_3	WT	Sacrificed
W_4	WT	Sacrificed
W_5	WT	Sacrificed
W_6	WT	B-lymphoma
W_7	WT	B-lymphoma
W_8	WT	B-lymphoma
W_9	WT	T-lymphoma
W_10	WT	T-lymphoma
W_11	WT	T-lymphoma
W_12	WT	Unknown (dermatitis)
W_13	WT	Retrobulbar tumour
W_14	WT	Retrobulbar tumour
W_15	WT	Cancer stomach
W_16	WT	Ovarian cancer
W_17	WT	Ovarian cancer

NPMc+ mice developed AML between 536 and 637 days with a median survival of 564 days (Figure 13B). Detailed histological analyses revealed that bone marrow samples of NPMc+AML mice showed an overall increase in cellularity, with a myeloid hyperplasia and signs of dysplasia of the erythroid and megakaryocyte series. The myeloid compartment was left-shifted, with an increase in immature cells and overall myeloblasts prevalence (Figure 14). Neoplastic aggregates and nodules composed by large cells with abundant cytoplasm and indented nuclei, morphologically suggesting a monocyte differentiation, were observed in extramedullary sites, including spleen, liver, kidneys and lungs (Figure 14). Blood smears were characterized by the presence of a high number of blasts (Figure 14). These animals displayed leucocytosis, thrombocytopenia, anaemia and splenomegaly (Figure 15) and the expression of the mutated NPM protein was clearly detectable in the infiltrated bone marrow derived cells (Figure 16).

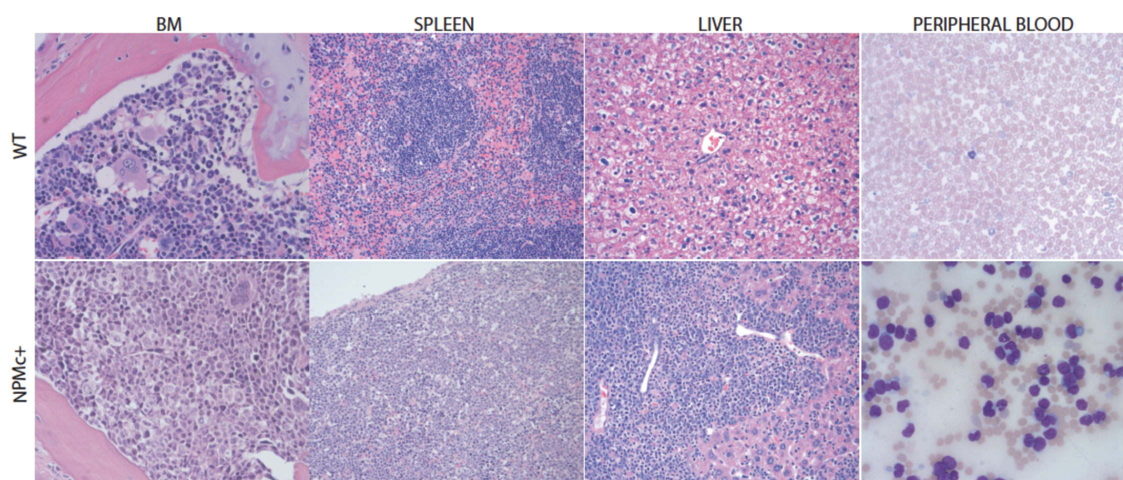


Figure 14 - Leukaemia development in NPMc+ BMT mice

Representative images of haematoxylin and eosin (H&E)-stained bone marrow (BM), spleen and liver section from NPMc+ leukaemic mice or control WT mice. May-Grunwald Giemsa staining of blood smears is shown in the right panel.

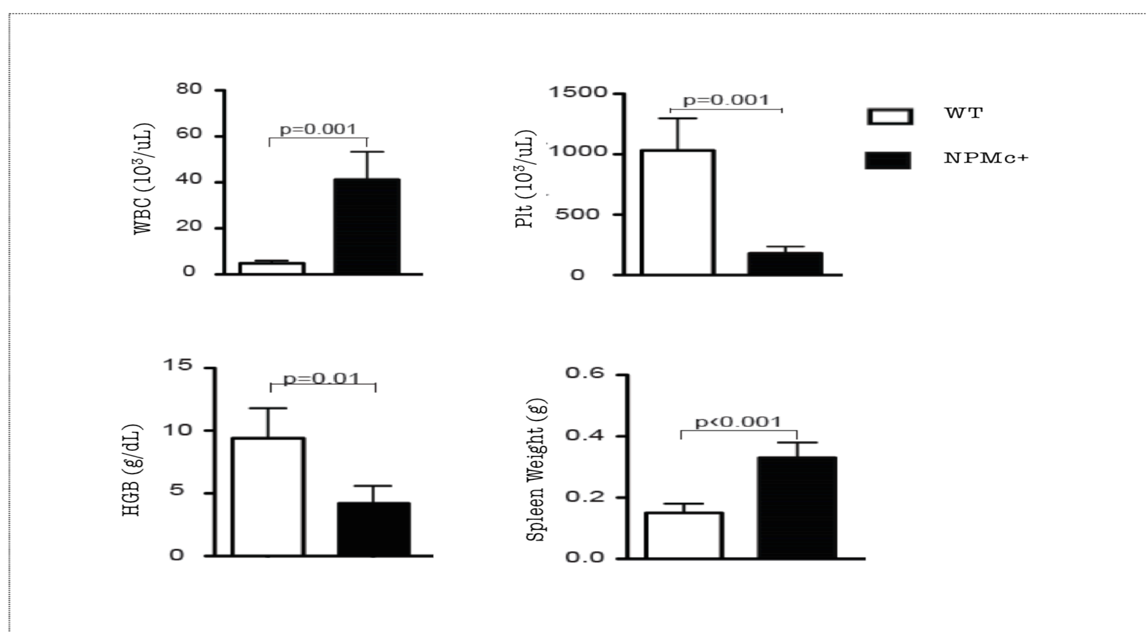


Figure 15 - Significant clinical alteration in leukemic NPMc+BMT mice.

A total of 50 uL of peripheral blood was collected from moribund mice along with samples from control mice and evaluated through an automated peripheral blood analyzer. The analysis showed leukocytosis (A), thrombocytopenia (B) and anaemia (C). Necropsy indicated splenomegaly (D). (NPMc+ N=5; WT N=5).

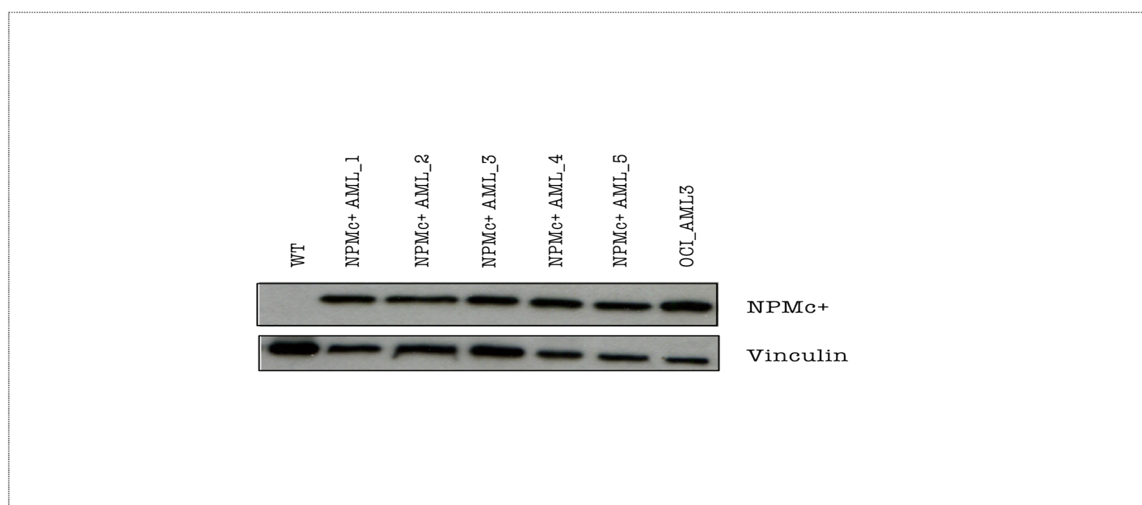


Figure 16 - NPMc+ protein is expressed in leukaemic blasts isolated from NPMc+ mice.

Western Blot analysis of NPMc+ protein expression with a mAb specific to NPM1mutA protein (T26) in blasts isolated from NPMc+ AML leukaemic mice. A NPMc+ positive OCI-AML3 cell line was used as positive control; BM cells isolated from TAT-Cre treated WT mice as used as negative control**.**

Moreover, leukaemic cells isolated from the spleen and transplanted into sub-lethally irradiated syngenic mice, led to the development of a fully penetrant secondary leukaemia sharing the same features with the primary lesion (Figure 17).

Altogether these data show that NPMc+ mutation is an initiating event leading to leukaemia development; however, the long latency and low penetrance of the disease, suggest that NPMc+ needs additional mutations to support a full-blown leukaemia.

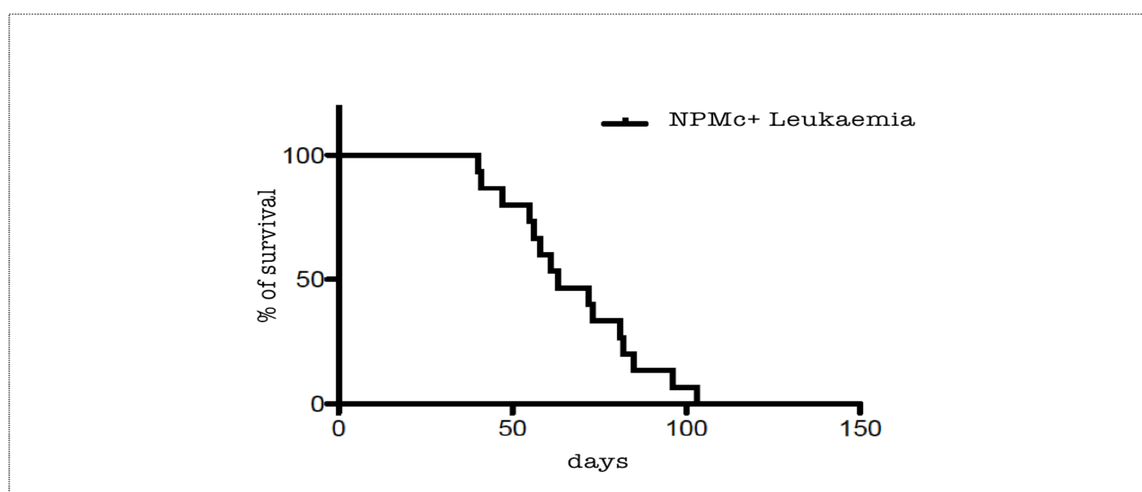
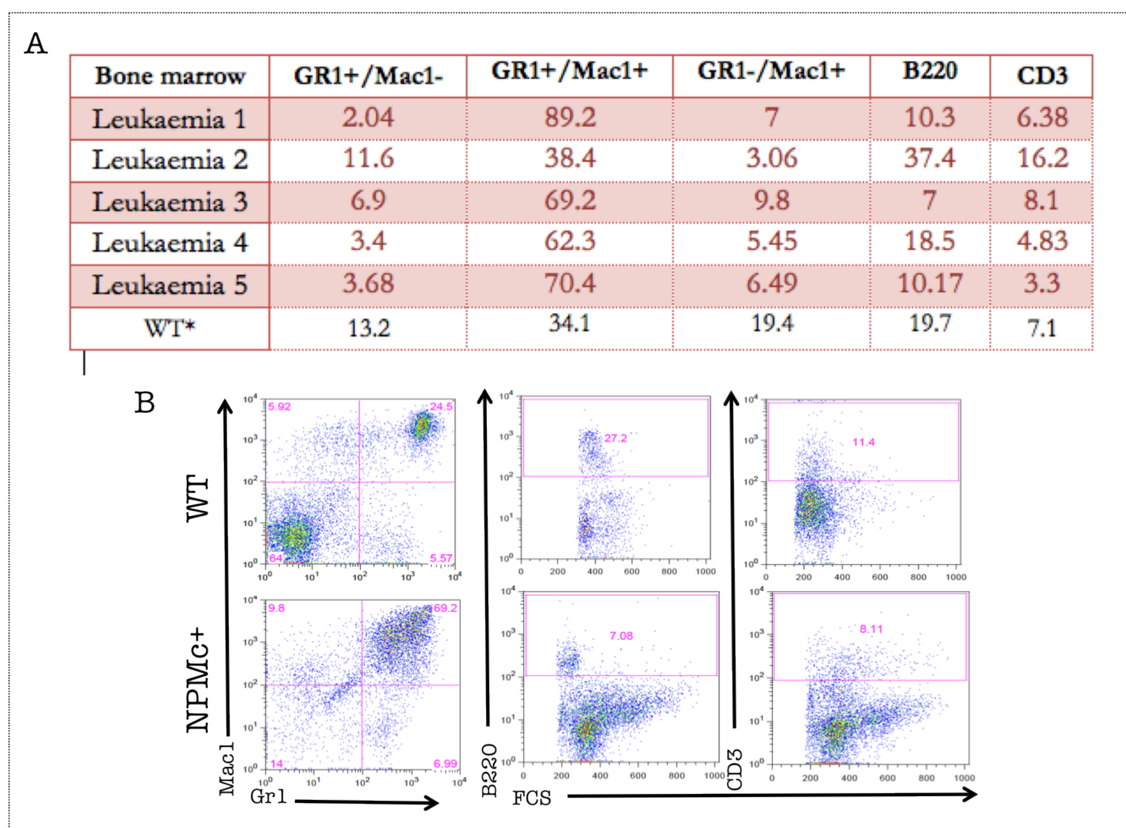


Figure 17 - NPMc+ leukaemia give rise to secondary leukaemia upon transplantation.

5 independent NPMc+ leukaemia were retranslated respectively into 3 recipient irradiated mice (N=15). The Kaplan-Mayer survival curve of the total pool of recipient mice is reported.

6.1.4 Immunophenotypic characterization of the murine NPMc+ leukaemia

To further characterize the murine NPMc+ leukaemia, FACS analysis of bone marrow samples were performed. The analysis showed that NPMc+ animals developed myeloid leukaemia with maturation (Mac1+/Gr1+) (Figure 18A-B) and in one case we observed a bi-phenotypic leukaemia with both myeloid (Mac1+/Gr1+) and lymphoid (B220+) lineage markers (Figure 18 A, Leukaemia 2).



A

Bone marrow	GR1+/Mac1-	GR1+/Mac1+	GR1-/Mac1+	B220	CD3
Leukaemia 1	2.04	89.2	7	10.3	6.38
Leukaemia 2	11.6	38.4	3.06	37.4	16.2
Leukaemia 3	6.9	69.2	9.8	7	8.1
Leukaemia 4	3.4	62.3	5.45	18.5	4.83
Leukaemia 5	3.68	70.4	6.49	10.17	3.3
WT*	13.2	34.1	19.4	19.7	7.1

Figure 18 - NPMc+ leukaemia immunophenotyping.

A Table showing immunophenotypic characteristic determined by FACS analysis of 5 NPMc+ leukaemia. B Representative plot showing flow cytometry determination of single cell suspension isolated from BM of a representative NPMc+ leukaemic mouse (AML3). An age matched WT mouse was used as control.

6.2 NPMc+ and FLT3-ITD mutations cooperate in inducing leukaemia development

The above results show that NPMc+ oncogene can initiates leukaemia, however, the long latency and low penetrance strongly suggest the requirement of cooperating mutations selection. The most frequent co-mutated gene in NPMc+ AML patients is *FLT3*. In particular, internal tandem duplications in this gene (ITD mutations) are found in 40% of NPMc+ AML (Falini et al, 2005), suggesting molecular synergisms. Furthermore, the mutational status of FLT3 gene has important prognostic factor for NPMc+AML patients, as mutations in FLT3 gene count for poor prognosis (Whitman et al, 2001)(Schnittger et al, 2002). In order to test the putative cooperation between the two genetic lesions in leukaemia development, we crossed the conditional NPMc+ mice with a constitutive heterozygote FLT3-ITD knock-in animal model (Lee et al, 2007) to generate double transgenic mice. The FLT3-ITD murine model, used in this study, has been described to develop a myeloproliferative disease that does not progress to acute myeloid leukaemia (Lee et al, 2007). BM-MNCs were isolated from double transgenic mice, treated *ex vivo* with TAT-Cre to induce expression of NPMc+ protein and transplanted into lethally irradiated CD45.1 syngeneic mice. Hereafter the BMT mice were referred to as NPMc+/FLT3-ITD mice. Strikingly, NPMc+/FLT3-ITD double mutant mice developed leukaemia very rapidly and with full penetrance. All NPMc+/FTL3-ITD mice died between 35 and 147 days post transplantation, with a median survival of 72 days (Figure 19). As previously observed in the NPMc+ leukaemic mice, splenomegaly, leucocytosis, anaemia and thrombocytopenia were found in the NPMc+/FLT3-ITD leukaemic animals (Figure 20).

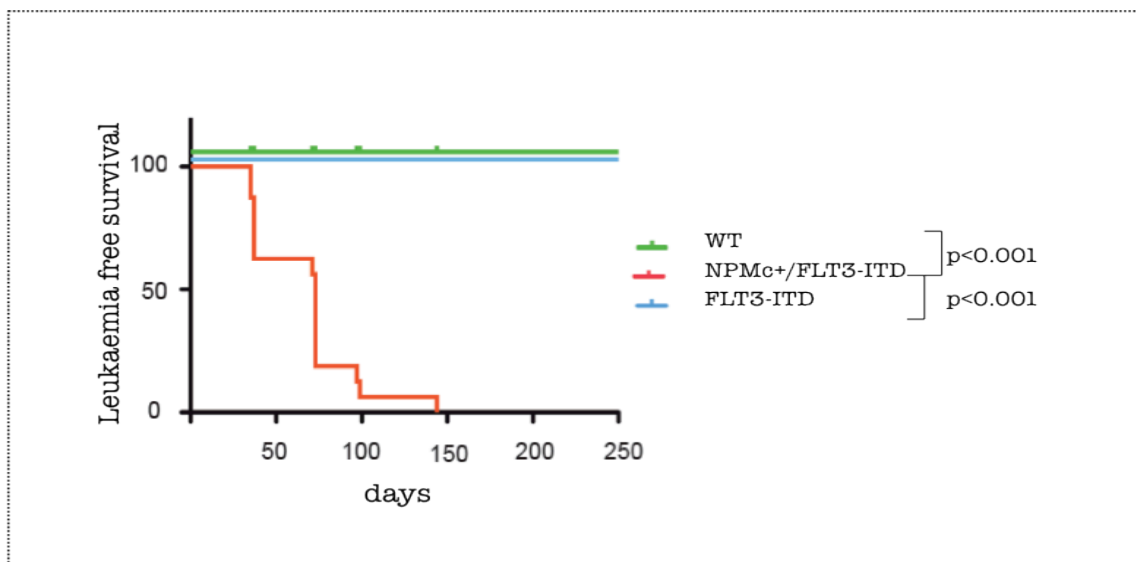


Figure 19 - NPMc+ and FLT3-ITD mutations efficiently cooperate to develop leukaemia.

Kaplan-Mayer survival curve of NPMc+/FLT3-ITD mice showing a potent synergism in leukaemia development. WT (N=16), FLT3-ITD (N=16) or NPMc+/FLT3-ITD (N=16) mice and treated with TAT-Cre.

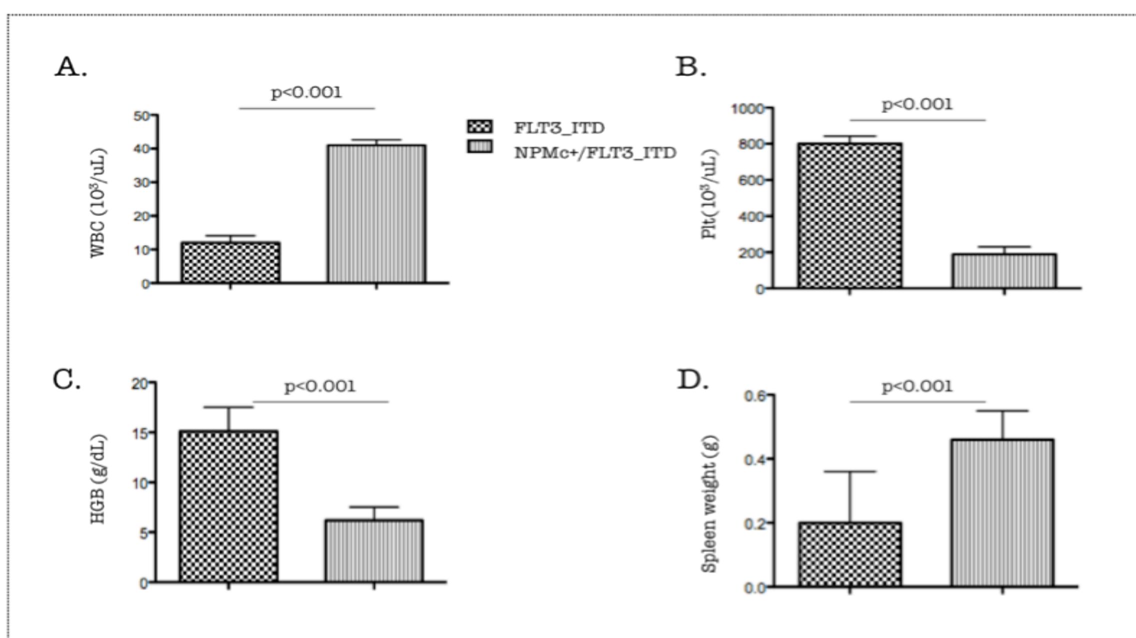


Figure 20 - Significant clinical alteration in leukemic NPMc+/FLT3-ITD BMT mice.

A total of 50 uL of peripheral blood was collected from moribund mice along with samples from control mice and evaluated through an automated peripheral blood analyzer. The analysis showed leukocytosis A, thrombocytopenia B and anaemia C. Necropsy indicated splenomegaly (D). (FLT3-ITD N=16; NPMc+/FLT3-ITD N=16).

Bone marrow histological inspection of NPMc+/FLT3-ITD mice, revealed a complete replacement of the hematopoietic series by a monotonous proliferation of blasts. In most of the cases, large neoplastic nodules composed of cells with the same features of those observed in the bone marrow were detected in extramedullary sites including spleen, liver, kidneys, lungs, gut and heart (Figure 21 for representative data). Blood smears were characterized by the occurrence of a high number of blasts.

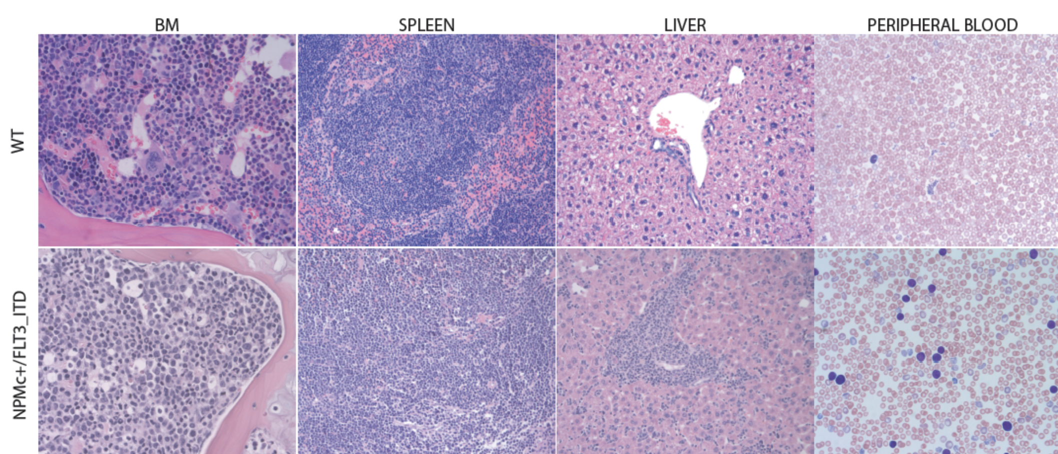


Figure 21 - Leukaemia development in NPMc+/FLT3-ITD BMT mice.
A total of 50 uL of peripheral blood was collected Representative images of haematoxylin and eosin (H&E)-stained bone marrow (BM), spleen and liver section from NPMc+/FLT3-ITD leukaemic mice or control WT mice. May-Grunwald Giemsa staining of blood smears is shown in the right panel.

Flow cytometry analysis of bone marrow from NPMc+/FLT3-ITD leukaemic animals showed that 62.5% of mice (10 out of 16) developed myeloid leukaemia with maturation (Mac1+/Gr1+), whereas 37.5% (6 out of 16) developed a biphenotypic leukaemia: 5 cases with both myeloid (Mac1+/Gr1+) and B-lymphoid (B220+) lineage markers and 1 case with both myeloid (Mac1+/Gr1+) and T-lymphoid (CD3+) lineage markers (Table 2).

The expression of the mutated NPM protein in the BM of leukaemic mice was confirmed by western blot analysis (Figure 22).

The short latency and the full penetrance of leukaemia emergence clearly demonstrate the striking cooperation between NPMc+ and FLT3-ITD mutations.

Table 2. Table showing immunophenotypic characteristic determined by FACS analysis of 16 NPMc+/FLT3-ITD leukaemia.

Bone marrow	GR1+/Mac1-	GR1+/Mac1+	GR1-/Mac1+	B220	CD3
Leukaemia_1	10.88	72.2	9.37	1.53	1.7
Leukaemia_2	23.8	55.5	8.8	8.09	10.7
Leukaemia_3	3.1	89.6	1.1	2.1	0.8
Leukaemia_4	5.6	83.6	4.3	0.5	5.2
Leukaemia_5	10.9	70.1	2.9	5.3	12.4
Leukaemia_6	8.3	58.9	8.9	49.9	0.1
Leukaemia_7	16.8	70.4	5.9	16.2	9
Leukaemia_8	11.1	45.4	23	58	3.3
Leukaemia_9	18.8	67	2.7	32.9	3.89
Leukaemia_10	2.6	74.5	12.6	3.8	4.6
Leukaemia_11	1.4	32.1	0.5	54.2	12.1
Leukaemia_12	3	13.2	0.1	2.5	51.7
Leukaemia_13	7.65	89.9	1.09	12.4	3.24
Leukaemia_14	9	24	2	27.3	0.15
Leukaemia_15	21.9	58.2	4.56	16.3	0
Leukaemia_16	21.7	42.5	13.3	39.3	12.3

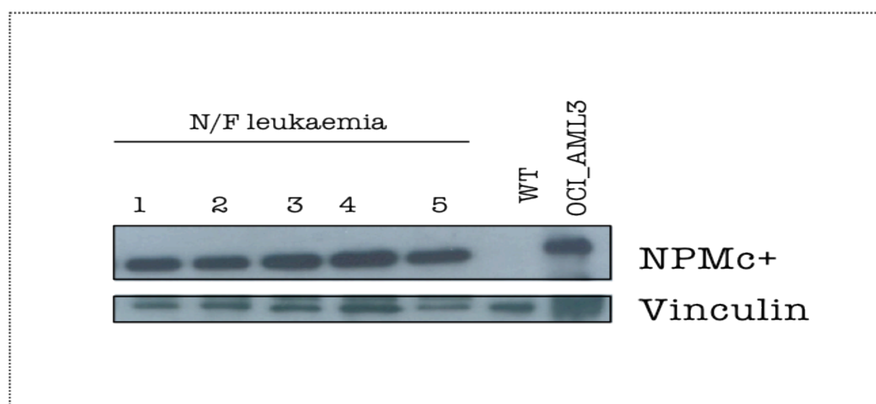


Figure 22 - NPMc+ protein is expressed in leukaemic blasts isolated from NPMc+/FLT3_ITD mice. Western Blot analysis of NPMc+ protein expression with a mAb specific to NPM1mutA protein (T26) in blasts isolated from 5 representative NPMc+/FLT3_ITD AML leukaemic mice (1-5 N/F leukaemia) . A NPMc+ positive OCI-AML3 cell line was used as positive control; BM cells isolated from TAT-Cre treated WT mice as used as negative control.

6.3 NPMc+ Lin- cells and NPMc+ leukaemic blast showed a Hox gene signature

The expression profile of patients with NPMc+AML has been previously correlated with the overexpression of homeobox-containing transcription factor genes (Alcalay et al, 2005). Therefore, to further validate our model, the expression of few *Hox* gene was evaluated both in lineage markers negative (lin-) cells isolated from pre-leukemic mice (3 months old) and in BM blasts derived from NPMc+ and NPMc+/FLT3-ITD leukaemic animals. Quantitative RT-PCR analysis confirmed that the same *Hox* gene found overexpressed in human NPMc+ AMLs were up regulated both in NPMc+ lin- pre-leukemic cells and in NPMc+ or NPMc+/FLT3-ITD leukaemic blasts (Figure 23).

This data confirms the capability of our mouse model to recapitulate unique features of NPMc+ AML. Moreover, the up-regulation of the *Hox* genes in the preleukemic phase indicates that the deregulation of the *Hox* gene is an early mark of NPMc+ expression in the more undifferentiated haematopoietic compartment and it may contribute to the leukemic process.

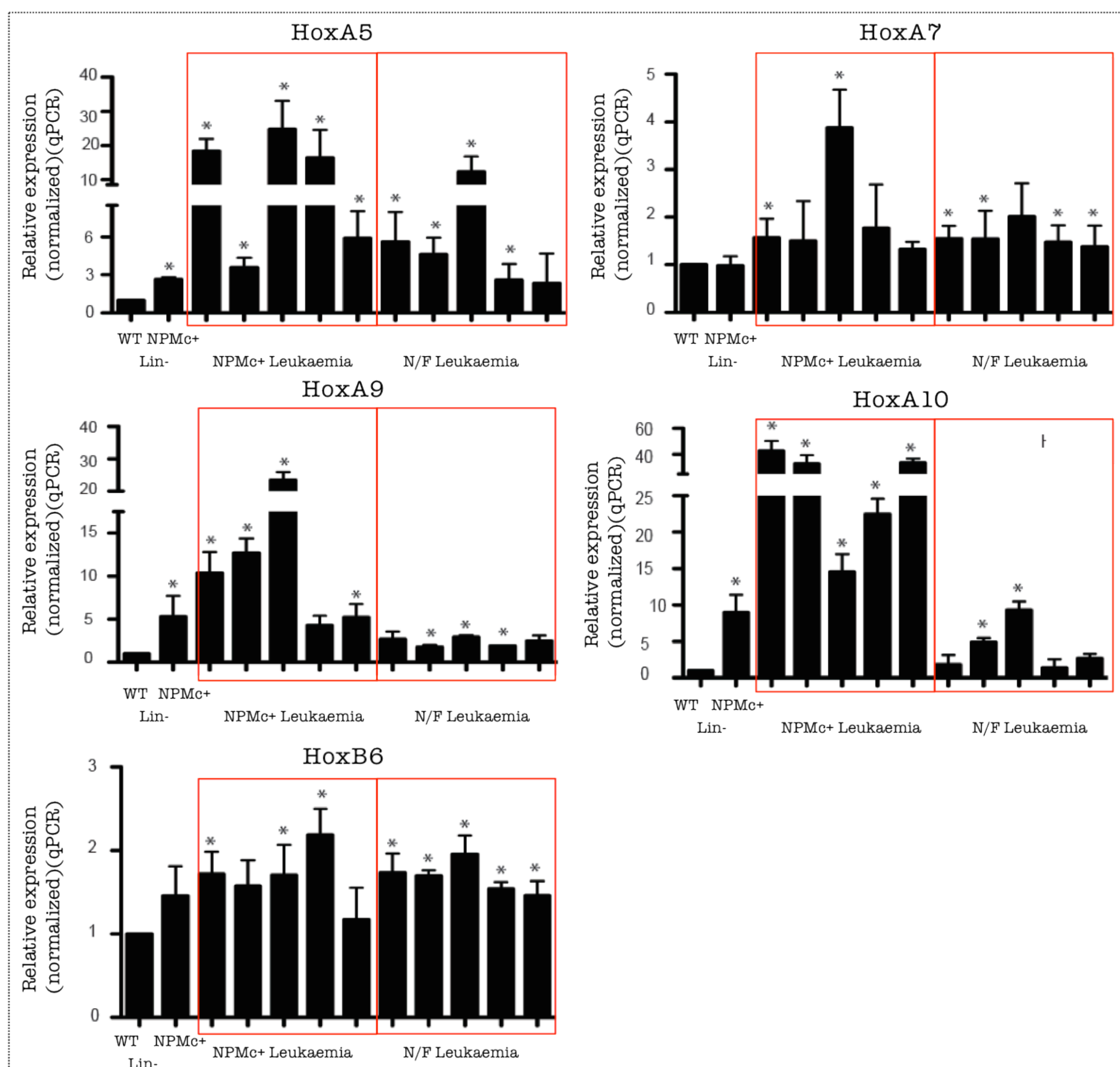


Figure 23 - HOX gene upregulation in NPMc+ lin-cells and NPMc+, NPMc+/FLT3-ITD leukemic blasts. Expression of Hox genes analysed by Q-PCR using mRNA from lin- cells isolated from TAT-Cre treated WT or NPMc+ BMT mice and from 5 NPMc+ AMLs and 5 NPMc+/FLT3-ITD (NF) AMLs . Expression was standardized with Tata Box Protein (TBP) and normalized against the control WT RNAs. *$p<0.05$

6.4 Biological impact of the NPMc+ expression in the haematopoietic stem and progenitor cell compartment

An open challenge to leukaemia field and, more in general, to cancer cell biology is the acquisition of a profound knowledge of the molecular pathways that induce the transition from normal to malignant cells and finally lead to the establishment of malignant clonal dominance. HSCs are thought to be the main target of early transforming events because they are long-lived cells with self-renewal ability and thus they can potentially fix and propagate initial mutations. The population of HSCs that harbour the early mutations, but not the entire set of alterations necessary to become frank leukaemia, could be referred to as pre-leukemic stem cells (pre-LSC). Pre-leukemic SCs, by definition, maintain the capability to differentiate into the full range of mature myeloid and lymphoid cells.

At present, the impact of cytoplasmic mutations of the *NPM1* gene on haematopoietic stem and progenitor cells compartment is completely obscure. In the present study, the NPMc+ mouse was used as a model system to investigate how the mutated NPM protein affects normal haematopoiesis and to study the underlying deregulated mechanisms that may be responsible for HSC transformation into LSCs.

6.4.1 Generation of NPMc+/YFP mice

TAT-Cre treatment of BM-MNCs leads to approximately 50% recombination efficiency in our experimental conditions (as reported in paragraph 6.1.2). Therefore, in order to track *in vivo* NPMc+ expressing cells, conditional NPMc+ mice were crossed with conditional ROSA26^{eYFP}reporter mice (Srinivas, 2001), which express the Yellow Florescent Protein (YFP) upon Cre mediated recombination. To test whether YFP signal directly correlates with NPM mutant protein expression, BM-MNCs isolated from double transgenic NPMc+/YFP mice, were subjected to TAT-Cre treatment and then FACS sorted into two distinct subpopulations: the YFP positive (YFP+) and the YFP negative (YFP-) cell sub-

fractions (Figure 24 A-B). Western blot analysis revealed that only YFP+ sorted cells expressed the mutant protein (Figure 24 C). This data indicates that TAT-Cre mediated recombination occurs both at the *yfp* and *npm* mutant transgenic loci with equal efficiency within the same cell. Therefore, YFP signal can be used as mirror for NPMc+ expressing cells in the NPMc+/YFP model system.

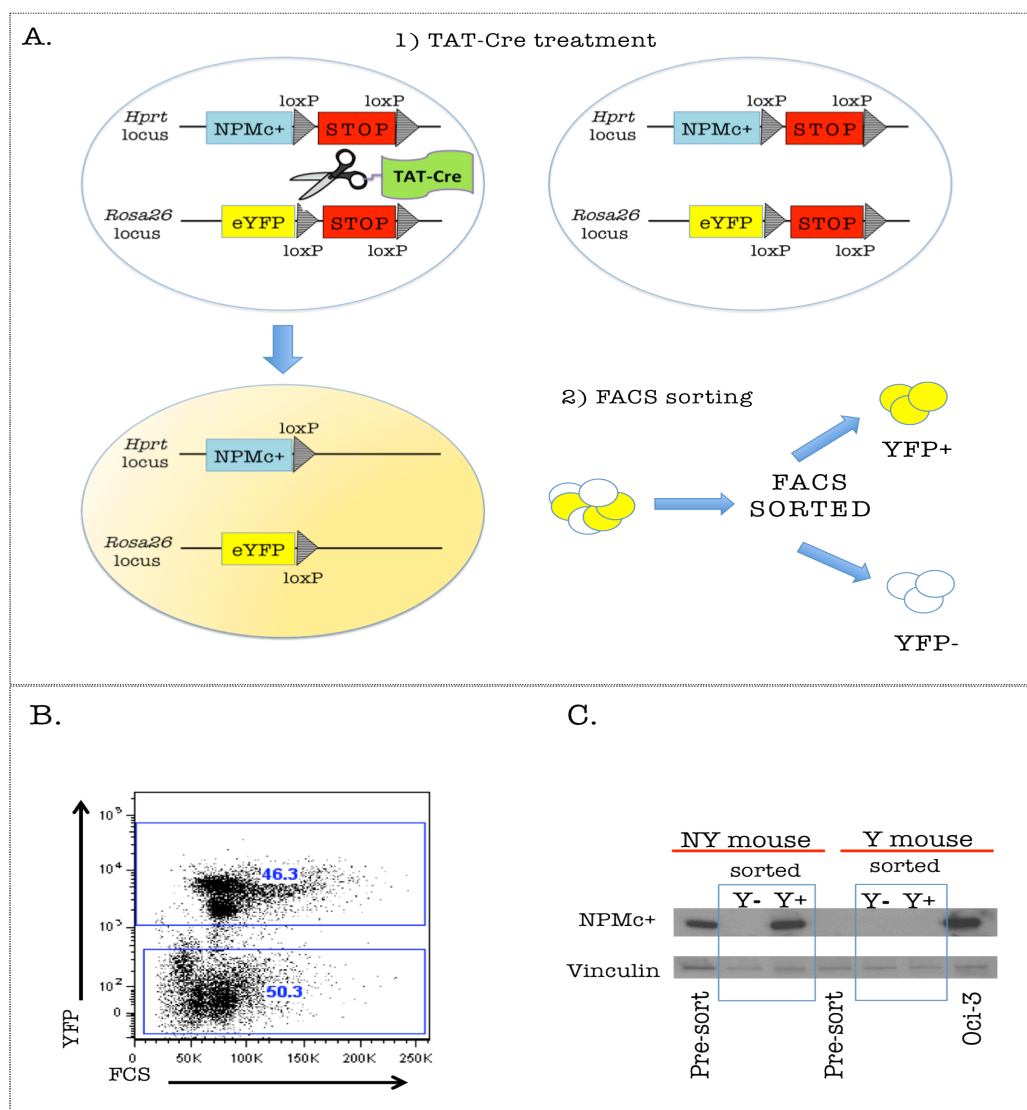


Figure 24 - YFP signal marks NPMc+ protein expression.

(A) Schematic representation of the experimental approach. BM-MNCs isolated from NPMc+/YFP mice were TAT-Cre treated (1). Recombined cells are depicted as yellow circles, whereas not recombined cells as white circles. The heterogeneous populations were FACS sorted according to YFP expression into YFP+ and YFP- subpopulations (2). The efficiency of the TAT-Cre mediated recombination at the *hprt* and *rosa26* loci was tested in in C. (B) Representative FACS plot showing gating strategy. (C) Western blot assay showing NPMc+ expression in the pre-sort sample, in the YFP- (Y-) and YFP+ (Y+) sorted fractions isolated from

NPMc+/YFP mice (NY). YFP- (Y-) and YFP+ (Y+) sorted fractions isolated from YFP mice (Y) were used as negative control for NPMc+ expression. NPMc+ positive OCI-AML3 cell line was used as positive control.

6.4.2 NPMc+ allele expression increases the haematopoietic reconstitution ability

HSCs supply all blood cells throughout life by making use of their self-renewal and mutilineage differentiation capabilities (Osawa et al, 1996). Therefore, they are crucial for long term reconstitution of haematopoiesis on transplantation into recipients with bone marrow ablation (Verfaillie, 2002). Short term haematopoietic reconstitution is exhausted prior to 4 months post BMT, therefore this time point is used to test long term regenerative potential (Morrison & Weissman, 1994).

To evaluate the impact of NPMc+ expression on HSC function during hematopoietic reconstitution, competitive BMT experiments were performed (Szilvassy et al, 1990). BM-MNCs were isolated from NPMc+/YFP mice or control YFP mice, TAT-Cre treated and YFP+ FACS sorted. Then, CD45.2 YFP+_WT or YFP+_NPMc+ expressing BM-MNCs were mixed in a 1:1 ratio with wild type CD45.1 competitor BM-MNCs and injected into lethally irradiated recipient mice. Mice were bled every 4 weeks and analysed for occurrence of CD45.2 chimerism in the peripheral blood. As shown in Figure 25, in mice transplanted with the NPMc+ expressing BM cells (red bars) we observed an increased percentage of YFP+ cells compared to the WT counterpart (white bars) suggesting an increased ability to outcompete the wild type CD45.1 BM cells and an increased capacity to contribute to the haematopoietic reconstitution.

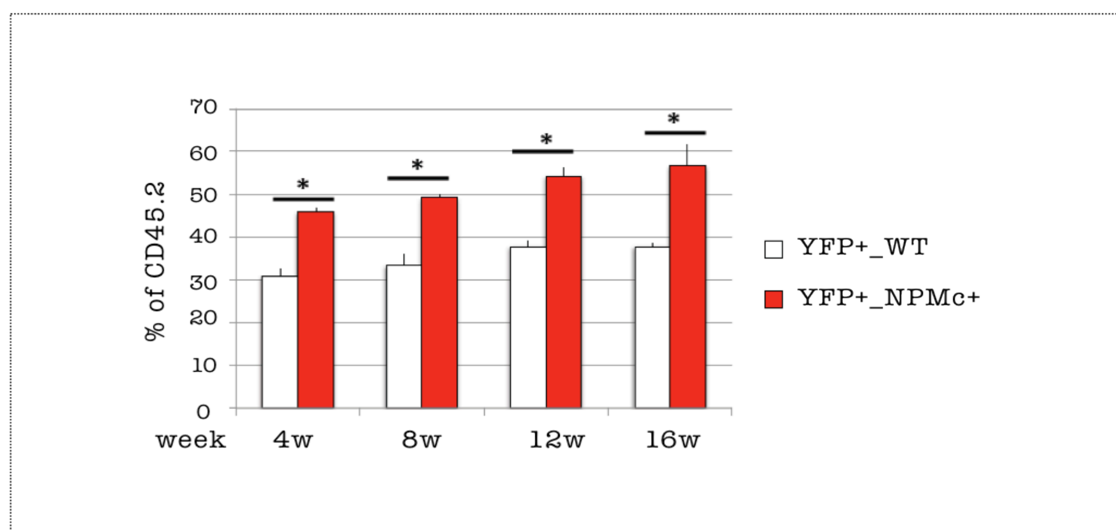


Figure 25 - Haematopoietic reconstitution potential of NPMc+ expressing BM-MNCs.
Percentage of CD45.2 in the peripheral blood of recipient mice (N=4) at different time points. The image referred to one of two independent experiments. * P value<0.05.

6.4.3 NPMc+ allele expression expands the pool of LKS and Long Term (LT)-HSC

The observed increased repopulating ability of NPMc+ HSC expressing cells, prompted us to investigate more in detail the haematopoietic stem/progenitor cell compartment (HSPC) in transplanted mice by immunophenotyping analysis. To address this point, mice 4 months post BMT were FACS analysed according to the HSPC unique cell surface profile (see materials and methods) (Spangrude et al, 1988) (Okada et al, 1992), (Figure. 26 A for representative results). The NPMc+ expressing BM population showed a significant increase in the total number of lineage negative, c-Kit+, Sca+ cells (LKS), which define the compartment containing both the long term (LT) and short term (ST) repopulating HSC and the multipotent progenitors (MPP). As we further dissected the LKS compartment based on the FLK and CD34 surface markers, we realised that both the LT and ST HSC are significantly expanded while no difference was appreciated in the MPP compartment (Figure 26 B). Interestingly, analysing the relative quantity of LT-HSC, ST-HSC and MPP in the LKS compartment, we observed a significant increase of the LT-HSC frequency and a

corresponding decrease in the MPP population (Figure. 26 C). These data collectively suggest that NPMc+ expression expands the most undifferentiated haematopoietic compartment and that this expansion is mainly due to its effect on the LT-HSC population.

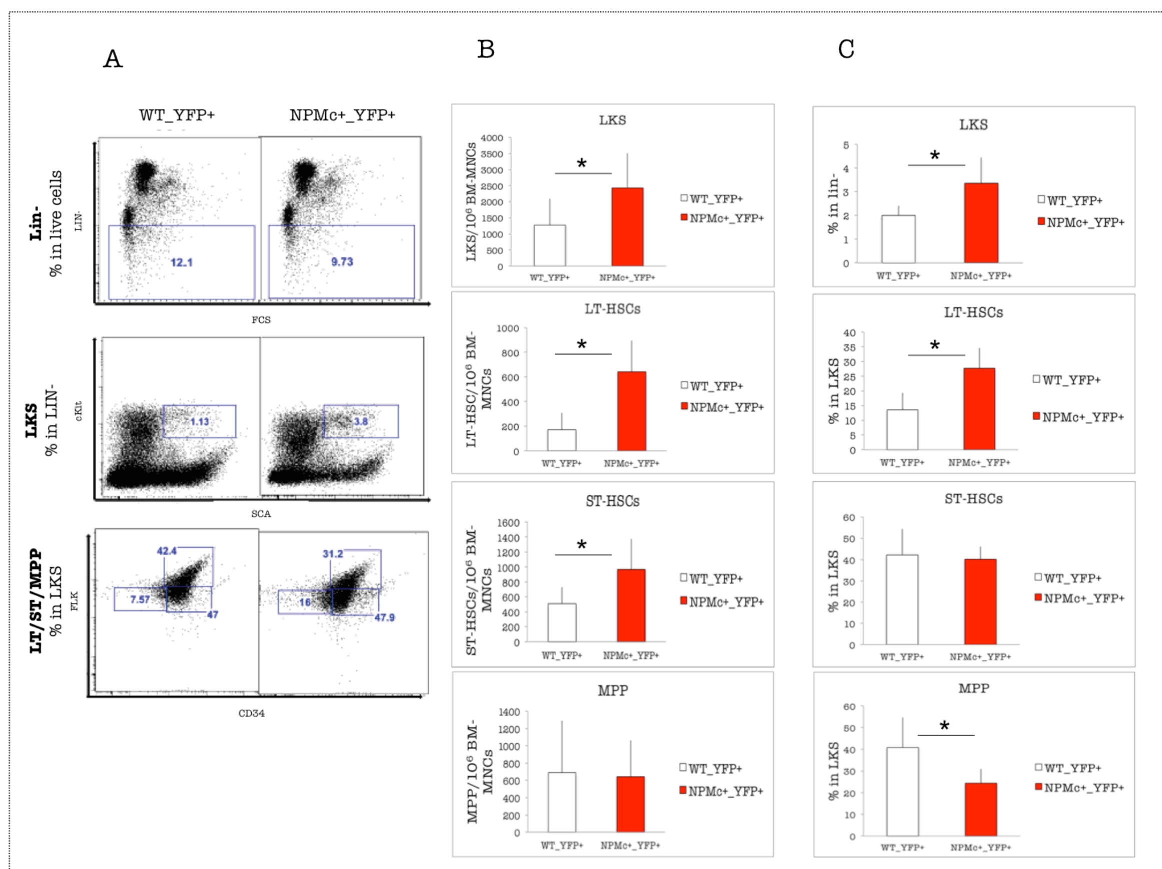


Figure 26 - HSC expansion in NPMc+ mice.

(A) Representative flow cytometric-gating strategy. Numbers (B) and frequency (C) of HSPC subpopulations in NPMc+ and WT BM. Images are the mean of 7 animals evaluated in 3 independent experiments. *p value <0.05

To confirm that the observed increased stem cells number was not due to an *in vitro* expansion of the HSC/progenitor population prior to the transplantation, NPMc+/YFP mice were crossed with a tamoxifen inducible Cre-ER^{TM} mouse strain (Hayashi & McMahon, 2002) to allow for inducible *in vivo* Cre recombination. Triple transgenic mice along with control Cre-ER/YFP mice were treated in vivo with tamoxifen in order to induce the translocation of Cre into the nucleus followed by Cre-dependent recombination.

Ten days after tamoxifen injection, BM-MNCs were purified and analysed as described in Figure 26 A to evaluate the frequency of HSPC and then transplanted in lethally irradiated syngeneic CD45.1 recipient mice. These mice were sacrificed 4 month after BMT and evaluated for their HSPC composition. This experiment allowed us to evaluate the frequency of NPMc+ expressing and WT HSPC prior and 4 months after BMT. As shown in Figure 27, after 4 months we could appreciate a significant expansion of the LKS and LT-HSC population only in the NPMc+ expressing HSPC.

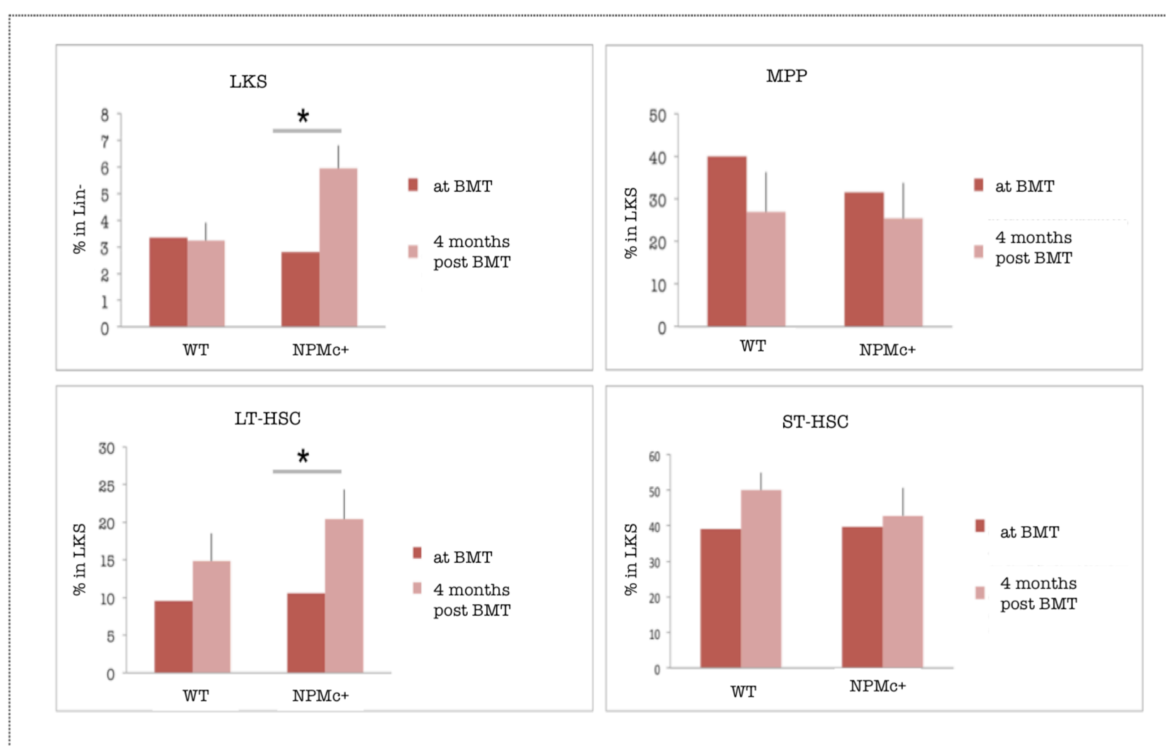


Figure 27 - HSC expansion in NPMc+ mice.

Summary graphs representing the frequency of LKS, LT-HSCs, ST-HSCs and MPP subpopulations in Cre-ER/YFP (WT) and NPMc+/ Cre-ER/YFP (NPMc+) mice at transplantation (dark red bars) and 4 month after BMT (light red bars). At transplantation N=1; 4 months post BMT N=5. *p value <0.05

6.4.4 NPMc+ expression leads to increased proliferation within normally quiescent LT-HSC compartment.

In the effort to determine the mechanisms of NPMc+ driven LKS and LT-HSC expansion, a possible hypothesis is that this oncogenic mutation increases cell proliferation. To evaluate the proliferation rate of WT and NPMc+ expressing BM cells we performed an *in vivo* BrdU incorporation assays (Passegue et al, 2005). Bone marrow was harvested 12 h post BrdU injection and stained both with anti BrdU and HSCP cell surface markers antibodies. The percentage of BrdU positive cells in each hematopoietic compartment was evaluated by FACS analysis. As shown in Figure 28, the NPMc+ expressing LKS compartment and its sub-populations (LT/ST-HSC and MPP) are characterized by an increased BrdU incorporation rate. These data support a putative role of NPMc+ in promoting cell cycle progression in the most undifferentiated hematopoietic compartment.

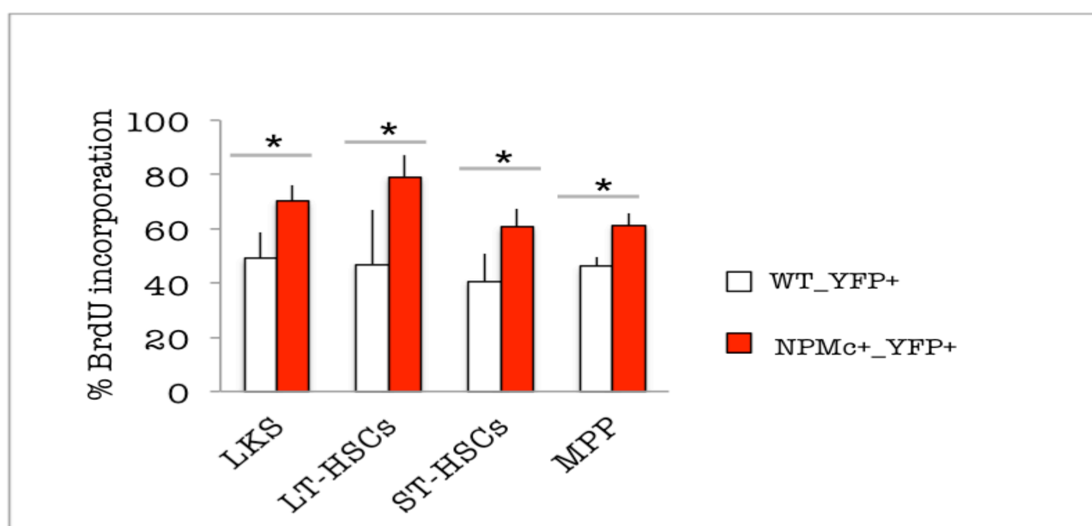


Figure 28 - NPMc+ HSPCs show increased proliferation.

BM-MNCs were harvested from WT_YFP+ and NPMc+_YFP+ transplanted mice 12 hr after BrdU injections and FASC analyzed according to the HSCP cell surface markers and the BrdU staining. . Percentage of BrdU positive cells in the LKS, LT-HSC, ST-HSCs and MPP compartments is reported. N=3.
*p value <0.05.

6.4.5 Higher proliferative rate in LT-HSC is not associated to loss of quiescent LT-HSC pool

Recent studies have shown that a small number of highly quiescent HSC have the greatest self-renewal potential and are responsible for haematopoietic reconstitution after injury, as upon treatment with the myelosuppressive agent 5-fluorouracil (5-FU) (Wilson et al, 2008). The increased proliferation rate of NPMc+ expressing HSCs raised the possibility that the quiescent pool of HSC might be recruited into cell cycle upon oncogene expression and eventually exhausted. To address this question, NPMc+ and control mice were intraperitoneally injected with 5-FU every 7 days to continuously deplete regenerating progenitor population. 5-FU kills cycling hematopoietic cells and challenges the quiescent pool of HSC to entry the cell cycle and to repopulate the depleted bone marrow (Harrison & Lerner, 1991) The survival curves of 5-FU treated animals showed increased resistance in NPMc+ expressing mice (Figure 29). This result suggests that, despite the increased proliferative rate in the presence of NPMc+, the reservoir of quiescence HSC is expanded and their self-renewal ability is maintained.

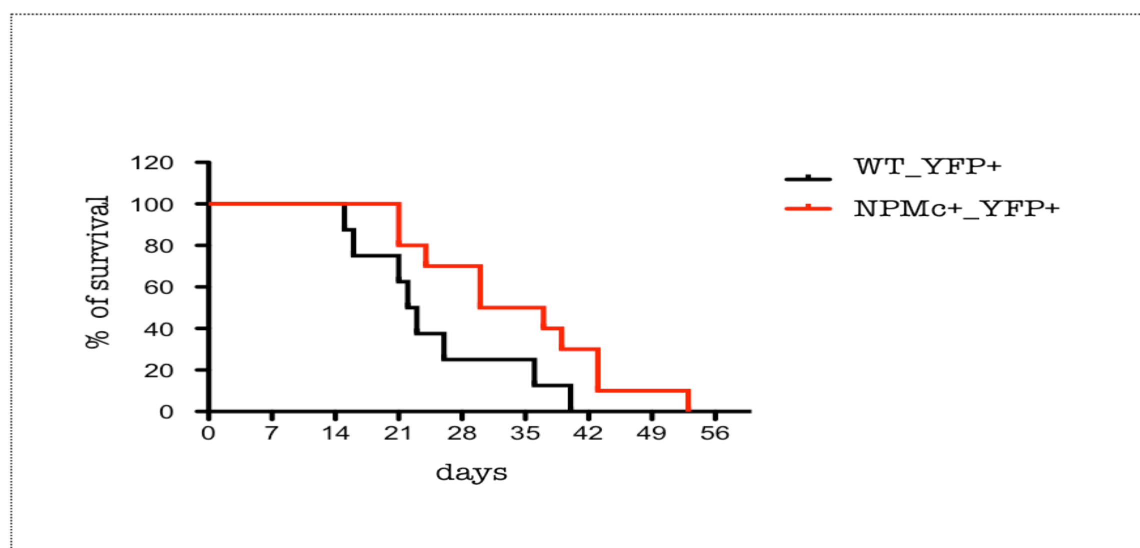


Figure 29 - NPMc+ mice show high survival to 5-Fu injection.

Kaplan-Mayer survival curve of control and NPMc+ mice injected intraperitoneally with 5-FU every 7 day.

P value<0.05

6.4.6 NPMc+ does not expand replicative potential in vitro

We have shown that NPMc+ expressing HSC have an increased proliferation rate, and as a consequence, the HSC pool is expanded yet maintaining the self-renewal and repopulating ability. Since the putative leukaemia initiating stem cell should be characterized by limitless self-renewal activity (Lane & Gilliland, 2010) we wanted to test if NPMc+ expression correlates with extended self-renewal ability. We have set up a serial transplantation experiment starting from the experimental scheme shown in Figure 24 A. CD45.2 NPMc+-YFP+ or WT_YFP+ sorted cells were transplanted in lethally irradiated CD45.1 mice, 4 weeks after BMT the peripheral blood was analysed and the extent of engraftment established evaluating the percentage of CD45.2 positive cells (not shown). Three months after BMT mice were sacrificed, CD45.2 bone marrow cells purified and re-transplanted in a second CD45.1 lethally irradiated recipient. In normal circumstances, such a protocol should be repeated until the death of the transplanted mice because of the progressive exhaustion of the HSC pool within the serial transplanted BM cells. Therefore, in this assay, the number of successful serial transplantation is an indirect measure of the self-renewal potential of the initial HSC pool. Unfortunately, in our hands after the third serial transplantation, mice transplanted with the NPMc+ expressing BM cells, developed leukaemia and died, preventing the completion of the assay (Figure 30).

As surrogate experiment we performed an *in vitro* serial replating assay in methylcellulose in the presence of IL3, IL6 and stem cell factor (SCF) cytokines. Equal number of YFP+ WT and NPMc+ expressing BM cells were purified from transplanted mice, plated and cultured for 7-10 days, then colonies were counted and cells were collected and replated. As shown in Figure 31 in the first plating we scored an increased number of colonies in NPMc+ expressing cells confirming the expansion of the more undifferentiated LKS compartment. By the second plating the difference in colony number between NPMc+ and WT cells was greatly reduced and both samples exhausted their *in vitro* replicative potential by the 7th

passage. Within the limitation of the assay, these data suggest that NPMc+ does not extend the replicative potential of HSCP cells *in vitro*.

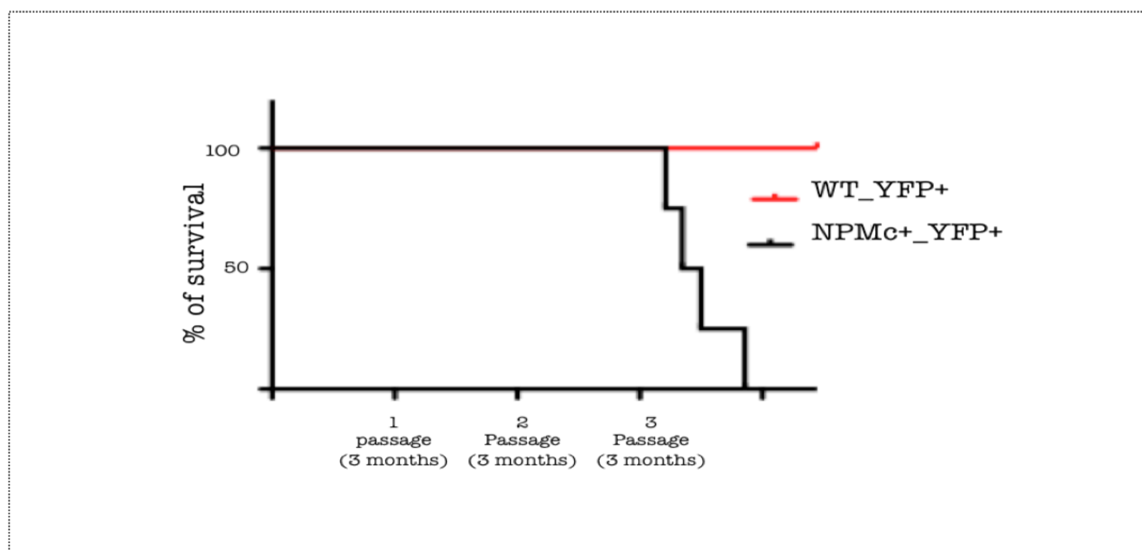


Figure 30 - Serial BMT accelerates leukaemia onset in NPMc+ mice.

Kaplan-Mayer survival curve of mice serially transplanted with NPMc+ BM-MNCs. BM-MNCs were harvested every 3 months, YFP+ sorted and then retransplanted in lethally irradiated CD45.1 mice. At third passage mice developed leukaemia.

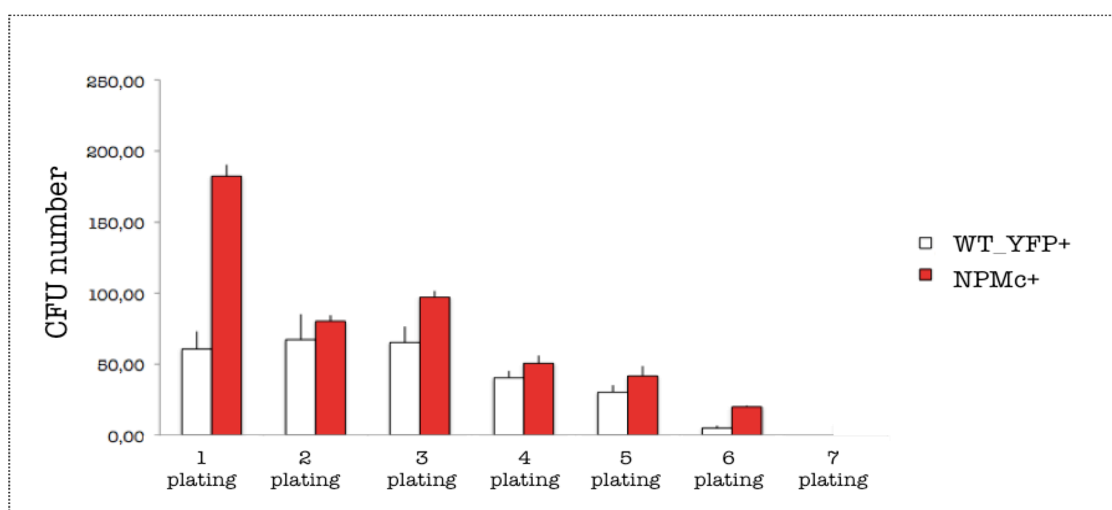


Figure 31 - NPMc+ increases the number of colony forming unit (CFU) but not expands replicative potential in vitro.

BM-MNCs from YFP+_NPMc+ and YFP+_WT transplanted mice were purified and serially plated on M3434 methylcellulose ((Lee et al, 2007). The number of colonies at each plating is reported.

7. Discussion

Acute Myeloid Leukaemia (AML) is a heterogeneous disease characterized by uncontrolled proliferation of myeloid blast cells and interference of the normal hematopoietic process. It represents the most frequent type of leukaemia in adults, with an estimated worldwide annual incidence of 3-4 cases per 100,000 people and a mortality of 4-6 cases per 100,000 people every year (Fey, 2009). The prognosis for patients with AML varies dramatically and is strongly influenced by a number of factors, including age, performance status, and, in particular, the karyotype at the time of diagnosis provides the most important independent prognostic information in adults with AML (Byrd et al, 2002). However, 40-50% of patients do not have clonal chromosomal aberrations (Mrozek et al, 2004). This group of cytogenetically normal AML is currently categorized as intermediate-risk group, although it needs more profound assessment (Tsimberidou & Estey, 2006) (Lowenberg et al, 2003). In recent years, the advent of progress in technology, as the high throughput sequencing methods, has provided the means for alternative approaches to the identification of disease alleles in NK-AML (Dohner, 2007) (Bullinger et al, 2004). Many of these patients carry recurrent genetic alterations that lead to changes in gene expression, such as mutations or polymorphisms (Schlenk et al, 2008). Somatic mutations in AML involve partial tandem duplications (PTD) of the *MLL* gene (Caligiuri et al, 1994), internal tandem duplication (ITD) (Nakao et al, 1996) or mutations in the tyrosine kinase domain (TKD) (Yamamoto et al, 2001) of the *FLT3* gene, mutations in the *NPM1* gene (Falini, 2005), *CEBPA* gene (Pabst et al, 2001), *NRAS* gene (Bos et al, 1985), *DNMT3A* (Yamashita, 2011) and *IDH1*(Mardis, 2009). In particular, the cytoplasmic mutations of the *NPM1* gene have been found to be the most frequent genetic alteration associated to adult NK-AML (60% of NK-AML and 35% of all AML) (Falini et al, 2005). Importantly, many of those mutations represent significant prognostic factors, allowing a more differential prognosis to help guide treatment choices (Schlenk et al, 2008). For instance, FLT3-ITD have been associated with short relapse-free and overall survival (Whitman et al, 2001)(Schnittger et

al, 2002), whereas a more favourable outcome is associated with cytogenetically normal cases of AML with mutations in *NPM* without concomitant *FLT3* alterations (Thiede et al, 2006). However, to formally demonstrate whether those mutations represent real driven events for tumour initiation or progression it is critical the development of accurate mouse models able to recapitulate the main feature of the human disease. Animal models provide incomparable insights into the mechanisms of leukaemia development and permit exploration of the molecular pathways of disease initiation, progression and maintenance in a more unbiased way then cell line (Ablain et al, 2013). Therefore, mouse models allow for the identification of potential new therapeutic targets and importantly they could be used as clinical purposes platform to test the efficiency of new therapeutic drugs.

The present PhD project has been developed in an effort to clarify the significance of the cytoplasmic mutant of the *NPM1* gene in leukaemia onset through the characterization of a novel conditional mouse model and the identification of the alterations induced by the oncogene during the pre-leukaemic phase preceding the evolution to the frank malignancy. We reason that those mechanisms are the key elements in NPMc+ driven transformation.

In our experimental setting, restricted hematopoietic expression of the most common *NPM1* genetic lesion leads to leukaemia onset in mice. This result definitively clarifies the relevance of NPMc+ oncogene expression to leukaemia onset and demonstrates that the mutated protein is an initiating lesion that prone to the establishment of the malignant clone.

The murine NPMc+ leukaemia share many features with the human NPMc+AML. First, based on morphological and immunophenotypic evaluation, the majority of the murine NPMc+ leukaemia could be classified as myelomonocitic/monocytic leukaemia, similar to the human M4-M5 AML subgroups according to the FAB classification. This characteristic is consistent with the fact that these subgroups are the most representative sub-types of the human NPMc+AMLs (Falini et al, 2005). In addition, a striking feature the human

NPMc+AML is the association to a unique gene expression profile characterized by up-regulation of numerous members of the homeodomain-containing family of transcription factors, including *HOX* and *TALE* genes (Alcalay et al, 2005). These genes are involved in the maintenance of a stem cell phenotype, since their expression is higher in HSCs and decreases during normal differentiation (Alharbi et al, 2013), (Magli et al, 1991). In accordance with these data, we found that NPMc+ murine blasts shown increased expression of these genes. Moreover, we observed that undifferentiated bone marrow cells (lineage marker negative cells, Lin-) isolated from NPMc+ expressing mice in the pre-leukaemic phases (3 months old mice) displayed a similar gene deregulation. The identification of this signature during the pre-leukemic phase suggests that the deregulation of a stem cell phenotype is one of the underlying abnormal mechanisms imposed by NPMc+ oncogene. The exact mechanism of the association between the *NPM1* mutation and the up-regulation of *HOX* genes is still unclear. A possible explanation is that NPMc+ directly disturbs the expression of *HOX* genes, or alternatively, that *NPM1* mutation arrest the differentiation of early hematopoietic progenitors in which *HOX* gene expression is up-regulated (Rau & Brown, 2009). Indeed, numerous studies have shown that deregulation of *HOX* genes is associated to haematopoietic malignancies. In this regards, first evidences came from the fact that their overexpression in mouse bone marrow leads to myeloproliferative disease and/or leukaemia. For instance, *Hoxa9* overexpression enhances HSC expansion and myeloid progenitor proliferation and, with a long latency, leads to leukaemia (Kroon et al, 1998; Thorsteinsdottir et al, 1999). In human leukaemia, *HOX* genes overexpression could be caused by chromosomal rearrangements that involve their upstream regulator such as MLL. Normally, MLL positively regulates the transcription of *HOX* genes by maintaining their expression through direct binding to a proximal promoter (Milne et al, 2002). MLL fusion proteins activate *HOX* gene transcription more efficiently then alone (Slany, 2009). As a consequence, myeloid differentiation is blocked

and proliferation is enhanced. Moreover, HOX genes can promote the development of AML by forming chimeric fusions with other genes, such as the NUP98 (Borrow et al, 1996; Nakamura, 2006).

Leukaemia onset represents the only significant variance between the survival curve of NPMc+ cohort and control animals, however, we also reported emergence of MDS cases in NPMc+ mice. Those animals died of severe anaemia showing hypocellularity in bone marrow with dysplastic features in the erythroid and megakaryocytic series (data not shown). Our observation raises the possibility of a more complex correlation between NPMc+ mutations and hematological disease pathogenesis. Indeed, while the occurrence of NPMc+ mutation in *de novo* AML is very well documented (Falini et al, 2005), its incidence in other myeloid malignancies remains less investigated, although it is clear the predominantly connection of this mutation to *de novo* AML cases. Recent investigations have tried to address in more detailed way the relevance of NPMc+ mutation to other myeloid disorders, as secondary AML (sAML) deriving from MDS or chronic phase of myeloproliferative disorders (MPD). Interestingly, Zhang and colleagues have reported occurrence of NPMc+ mutation in primary MDS cases with an incidence of 5.2% (Zhang et al, 2006). This recent finding confers additional value to our observation of MDS cases associated to NPMc+ expression in our murine model, underlining the capability of our model system to recapitulate main pathological consequences of the NPMc+ expression into the hematopoietic compartment. Additional study by Schnittger and colleagues tried to address the relevance of NPMc+ in myeloid disease progression by back-tracking NPMc+ mutation state at diagnosis of MDS or MPD cases and at the time of progression in the leukaemic phase (Schnittger et al, 2011). This study suggests that NPMc+ can be present at diagnosis of MDS or MPN or can emerge later during the follow-up of the disease in addition or in parallel with other molecular or cytogenetic alterations. This supports the idea of a multistep transforming process from MDS or MPD to s-AML and suggests that

NPMc+ is not only a key factor in the initiation of *de novo* AML but may contribute to MDS or MPD and its progression to sAML (Dohner et al, 2005). Additional evidences suggesting a role of NPMc+ mutations during pre-leukaemic lesion, as myelodysplastic syndrome, come from another recent paper reporting a novel NPMc+ conditional mouse model (Sportoletti et al, 2013). In this model, the NPMc+ cDNA is inserted into the *Rosa26* locus and the CAG promoter drives the expression of the mutated protein. To induce expression of the mutated protein, NPMc+ mice were crossed with Mx-Cre mice and double transgenic animals were treated with pIpC to induced Cre expression followed by recombination at loxP sites. This model shares similar genetic design with our system. Both *Hprt* and *Rosa26* locus are characterized by open chromatin structure and can guarantee transgene expression while avoiding the drawbacks of random insertion, transgene silencing, and the deregulation of neighboring genes, making them widely used for the generation of knock-in mouse model. Remarkably, Sportoletti's model does not develop leukaemia late in life but displays megakaryocytes expansion and block of differentiation, which mirror some of the dysplastic features in the megakaryocytes series found in the bone marrow samples from human NPMc+ AML patients (Sportoletti et al, 2013). Therefore, also this model suggests a possible involvement of NPMc+ mutation in inducing myelodysplastic features, which in human frequently represents a pre-leukaemic phase (Heaney, 1999). Although genetic similarity between our and Spartoletti's model, two different main phenotypes have been reported. A likely explanation for this divergence may be the presence of a cooperating event for leukaemia development such as the hyper-proliferation induced thought bone marrow transplantation in our system. Indeed, our results show that bone marrow transplantation cooperates for leukaemia onset in NPMc+ driven leukemogenesis (acceleration of leukaemia development and increased penetrance upon serial transplantation experiment).

Altogether these observations support the reliability of our system and its value as platform to study the pathogenesis of the NPMc+ dependent leukaemia *in vivo*. However, the leukaemia onset in our model is characterized by a long latency and low penetrance. This data are consistent with another recently published NPMc+ knock-in mouse model (Vassiliou et al, 2011). Vassiliou's mouse model is of particular interest as it entirely mimics the genetic lesion that human patients harbour. The conditional allele was designed into the native murine *NPM* locus, hence, mice expressed only one copy of the wild type NPM protein and one mutant protein driven by the same endogenous promoter. Also, in this case mice developed leukaemia late in life with a low penetrance (about 30% of the animals) and about 50% of the leukaemia were bi-phenotypic (B/myeloid). The long latency and low penetrance suggest that NPMc+ needs additional mutations to support a full leukaemia phenotype, and that not all the animals acquire such mutations during their life span. Indeed, in the same paper, Vassiliou et al. also investigated the occurrence of cooperative mutations through transposon insertional mutagenesis. Among others, mutations in the *Flt3* gene have been selected; however, under their experimental conditions, they isolated *Flt3* mutations both in NPMc+ and WT mice, thus leaving open the question if FLT3 and NPM mutations significantly cooperate in leukaemia development. To address this issue we have crossed our NPMc+ model with a constitutive knock-in FLT3-ITD mouse model (Lee et al, 2007). FLT3-ITD animals have been shown to develop myeloproliferative disorder that did not progress to leukaemia. NPMc+/FLT3-ITD mice, instead, developed leukaemia very rapidly. Strikingly, all the NPMc+/FLT3-ITD mice died of leukaemia between 35 and 147 days with a median survival of 72 days. Our data demonstrate that NPMc+ and FLT3-ITD mutations cooperating powerfully in inducing AMLs in mice. This finding is even more remarkable in light of the kinetic of leukaemia development in NPMc+/FLT3-ITD animals compared to the relatively moderate effects on murine haematopoiesis that mice bearing only one of the two

mutations showed. The extremely rapid onset of disease would suggest that NPMc+ and FLT3-ITD mutations when combined are enough to initiate and promote leukaemogenesis. Alternatively, FLT3-ITD mutation provides proliferation and survival signalling that increase the occurrence of cooperating mutations. The whole genomic sequencing of NPMc+ and NPMc+/FLT3-ITD leukaemia should help in verifying these alternative hypotheses. It has been proposed that AML is the consequence of the cooperation of two functional classes of mutations: class I mutations, conferring a proliferative and/or survival advantage, and class II mutations, which impair hematopoietic differentiation and/or self renewal proprieties of the stem cell progenitors (Haferlach, 2008), (Welch et al, 2012). Our murine model strongly supports this hypothesis with NPMc+ being the initiating event that confers clonal advantage and FLT3-ITD a strong cooperating mutation. It will be intriguing to verify whether NPMc+ acts synergistically also with other recurrent mutations associated to NPMc+AML, as DNMT3A or IDH1 (Takahashi, 2011).

A major challenge for this study is to identify critical mechanisms imposed by the NPMc+ oncogene activation on the normal haematopoiesis and that could count for the establishment of the malignant clone and the leukaemia development. Because tumours can be considered as hierarchically organized tissues, it has been proposed that, a subset of cells with analogous properties of normal stem cells (SC) is responsible for the sustenance of the malignant cellular mass (Reya et al, 2001) (Shipitsin & Polyak, 2008). This observation has been postulated in the cancer stem cell theory. The strong similarity between normal and cancer stem cell makes reasonable to proposed that CSC derived from transforming events in the pool of normal stem cell, or by the aberrant acquisition of stem cell proprieties in progenitor subset (Tan et al, 2006). Therefore, one of the first possibilities that we investigated was whether NPMc+ expression affects the biology of the HSCP compartment during the pre-leukemic phases. This investigation has allowed us to

make several important observations. Our data show that the expression of the NPMc+ oncogene in HSPC compartment expands the most undifferentiated haematopoietic pool, and in particular the LT-HSC fraction. We found that one of the putative mechanisms responsible for this expansion is an increased proliferation rate in the entire LKS compartment. Interestingly, despite we did not observe any difference in MPP cell number, we detected also in this population a higher BrdU incorporation rate. This apparent discrepancy could be explained considering additional contribution of NPMc+ mutant protein on survival pathways (e.g. increased apoptosis) or, alternatively, NPMc+ expression may influence the differentiation process in the progenitor compartment. Indeed, we have observed a significant increased number of mature myeloid cell (MAC1+/GR1+) cells both in the bone marrow and in the spleen of NPMc+ animals (data not shown).

The replicative history of the HSCs correlates to their biological properties and in particular to their self-renewal ability (Orford & Scadden, 2008; Wilson et al, 2008). Indeed, the hyper proliferation imposed by serial transplantation results in the premature functional exhaustion of the initial HSCs pool (Kamminga et al, 2005). The cellular signal that controls the rounds of HSCs replication is probably related to a progressive accumulation of DNA damage in the cell (Viale et al, 2009). In a recent study Trumpp and colleagues demonstrated the existence of highly quiescent SCs (dormant stem cells) among immune-phenotypically identical but actively cycling HSCs (Wilson, 2008). Importantly, this dormant population is shown to possess the highest self-renewal potential and is essential to replenish the haematopoietic system upon stress and injury, thus preventing HSC exhaustion. The hypothesis is that dormancy is the mechanism through which HSCs preserve their self-renewal potential. Our 5-FU experiments in mice 4 months after BMT strongly suggest that despite the increased proliferative rate in the NPMc+ expressing HSCs compartment, not only they were not loosing their self-renewal abilities, but they showed an expanded capacity of repopulating the haematopoietic compartment upon

ablation. This results can be explain both by the expansion of the quiescent HSCs pool or by an extended self renewal abilities. The latter is an important acquired feature of cancer stem cells (CSC). As we found the pool of HSC expanded, we asked whether NPMc+ is able to expand also the replicative potential of the HSCs. The gold-standard experiment to test if the HSC pool displays enhanced self-renewal abilities is the *in vivo* serial transplantation assay (Viale et al, 2009). In our experimental setting, this assay ended up with an accelerated and full penetrant leukemogenesis in animals expressing NPMc+. This result prevented any conclusion about a putative NPMc+ dependent extended self-renewal, however, allowed us to conclude that the increased proliferation of the HSC compartment imposed by serial round of repopulation of the haematopoietic compartment is, *per se*, a cooperating mechanism of leukemogenesis *in vivo*. The molecular mechanism underling this cooperation needs to be explored but it could be related to the reported oncogene associated replicative DNA damage (Di Micco et al, 2006), thus leading to an increased rate of mutations in cooperative genes. Also in this case, whole-genome sequencing of theses leukaemia would help in understanding if the mutation rate had increased. Moreover, although indirectly, these data suggest that the increased proliferation rate imposed by NPMc+ in the HSCs compartment may be one of the mechanisms by which NPMc+ promote leukaemia. Going back to our original question about the possibility that NPMc+ could expand the proliferative potential of the HSCs, the in vitro serial replanting assay indicate that NPMc+ HSPC cells do not enhance their replicative potential that exhausts at the same rate as control cells. This *in vitro* data is further supported by the observation that when we analysed the HSPC compartment in mice at a later time point (8 months post BMT) we found an attenuation of the LT-HSC expansion and, in same cases, even a reversion of this phenotype with a reduction of the number of LT-HSCs (data not shown). These data suggest that NPMc+ is not able to enhance the number of cell divisions that a normal HSC can perform during its life span. Interestingly, as we reported in the results

section, we found two cases of MDS in the NPMc+ cohort of mice. MDS is a group of disorders in which the HSCs are defective and undergo to functional exhaustion resulting in few and poorly functioning blood cells (Pang et al, 2013). Therefore, we can hypothesize that the in the MDS mice (that notably died by severe anaemia) we experienced a progressive loss of self-renewal ability of the HSCs due to an abnormal proliferative rate.

Based on the reported results, we can start to depict a scenario about the effect of NPMc+ on the normal haematopoietic compartment and the underling mechanisms leading to leukaemia development. NPMc+ expands the pool of fully functioning HSCs, however, the higher proliferative rate may eventually causes a progressive depletion of the HSC compartment leading to bone marrow failure syndromes. Stochastically, the higher proliferative rate (maybe in combination with other NPMc+ cellular activities) favours the selection of mutations in cooperating genes that eventually expand the HSCs cell renewal activity further promoting the selection of a starting leukemic clone.

In this scenery it would be interesting to understand which is the contribution of FLT3-ITD mutations to leukaemia development. In a recent paper Chu et al. investigated the impact of this mutation to the HSPC through the characterization of a knock-in mouse model (Chu, 2012). They reported that physiological expression of FLT3-ITD mutation results in changes in the HSPC composition, with reduction of the LT-HSC sub-fraction associated to an increase in the proliferation rate. In accordance to the depletion of the more primitive stem compartment, they found functional defects in haematopoietic reconstitution capacity of FLT3-ITD BM cells. These data suggest that the putative mechanism of leukemiogeneis sustained by FLT3-ITD is the increased proliferative rate of the HSPC. Therefore, it is suggested that FLT3-ITD mutations do not conferred extended self-renewal, but act in a pathway similar to the one that we described for the NPMc+ oncogene. On the other hand, our data still suggest that NPMc+ transformation is favoured by abnormal proliferation as it is suggested by acceleration of leukaemia onset in

serial NPMc+ BMT mice. Thus, it seems that, the strong cooperation between NPMc+ and FLT3-ITD mutations could not be explained with a contribution of FLT3-ITD in enhancing the self-renewal potential of HSPC. However, it could not be excluded that the two mutations together acquire novel functions. Dissecting the molecular mechanisms of the powerfully cooperating potential of NPMc+ and FLT3-ITD mutations would give valuable insight into our knowledge of the leukaemia pathogenesis.

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