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**Modulation of stem cell signalling pathways by  
nucleophosmin and its leukemogenic mutant**

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“The fact that we live at the bottom of a deep gravity well, on the surface of a gas covered planet going around a nuclear fireball 90 million miles away and think this to be normal is obviously some indication of how skewed our perspective tends to be.”

– Douglas Adams, *The Salmon of Doubt: Hitchhiking the Galaxy One Last Time*





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## LIST OF ABBREVIATIONS

AML	acute myeloid leukaemia
NPM1	nucleophosmin
NPMc+	cytoplasmic mutant nucleophosmin
mRNA	messenger RNA
DKK1	dickkopf-related protein 1
HSC	hematopoietic stem cell
FACS	fluorescence activated cell sorting
LT-HSC	long term hematopoietic stem cell
ST-HSC	short term hematopoietic stem cell
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
PCP	planar cell polarity
hpf	hours post fertilization;
SBmo	splice-blocking morpholino
CE	convergence and extension
DAB	3,3'-diaminobenzidine
Tris	tris(hydroxymethyl)aminomethane

EDTA	ethylenediaminetetraacetic acid
PBS	phosphate buffered saline
atRA	all trans retinoic acid
GM-CSF	granulocyte-macrophage colony-stimulating factor
NBT	nitro blue tetrazolium chloride
BCIP	5-bromo-4-chloro-3-indolyl phosphate
DTT	dithiothreitol
qPCR	quantitative polimerase chain reaction
RT	room temperature
SDS	sodium dodecylsulfate
RIPA buffer	radioimmunoprecipitation assay buffer
GFP	green fluorescent protein
BrdU	5-bromo-2'-deoxyuridine
SCF	stem cell factor
IL-3	interleukin 3
PKC	protein kinase C
IP	immunoprecipitation

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## ABSTRACT

About one third of acute myeloid leukemias (AML) is characterized by the aberrant cytoplasmic localization of nucleophosmin (NPM1), a ubiquitously expressed phosphoprotein that acts as a molecular chaperone and shuttles between nucleus and cytoplasm. Although several animal models have been generated to unravel the mechanism of action of the cytoplasmic mutant NPMc+, it remains poorly understood.

In this thesis, we identified a novel function of both wild type NPM1 and NPMc+ in the modulation of Wnt signaling during zebrafish development and primitive hematopoiesis. The injection of *NPMc+* and human *NPM1* mRNAs in one cell stage zebrafish embryos reveals an opposite effect of the two proteins in the modulation of the Wnt signaling: NPM1 can inhibit the pathways whereas the mutant can activate it. Furthermore, NPM1 and NPMc+ have an opposite effect on the expression of *dkk1b*, a well known inhibitor of the Wnt pathway, and the co-injection of *NPM1* and *NPMc+* mRNA rescues the phenotype, suggesting a dominant negative effect of the mutant on the wild-type. Through whole mount in situ hybridization, markers of hematopoiesis have been studied revealing that the myeloproliferative effect of NPMc+ can be overcome by the co-injection of *dkk1b*, suggesting that the mutant can act by activating the pathway.

Moreover, we generated an NPMc+ expressing mammalian in vitro system using a non-transformed hematopoietic/progenitor cell line (EML-C1). Although NPMc+ is strongly and stably expressed in EML-C1, we did not observe any phenotype or alteration in Wnt signaling.

Taken together, data presented in this thesis showed that NPMc+, the leukemogenic mutant of NPM1, is able to act in a dominant negative fashion on NPM1 and displays a myeloproliferative effect during primitive zebrafish hematopoiesis. We showed that the proliferative effect of NPMc+ can be overcome by the simultaneous inhibition of the Wnt pathway through overexpression of *dkk1b*, suggesting that NPMc+ can activate Wnt signaling and that the pathway may be involved in the mechanism of NPMc+ AML establishment and/or progression.

## **INTRODUCTION**



## 1. HEMATOPOIESIS

Hematopoiesis is the process through which hematopoietic stem cells (HSC) give origin to all mature blood cells. These can be divided in two groups: myeloid and lymphoid lineages. The first group includes erythrocytes, platelets, monocytes, granulocytes (i.e. neutrophils, eosinophils and basophils) and mast cells, the second gives rise to natural killer (NK) cells, B and T lymphocytes. Dendritic cells can derive from both the myeloid and the lymphoid lineages [1].

HSCs are multipotent cells, able to divide asymmetrically, giving origin to another HSC (self-renewal) and a committed progenitor that can differentiate toward the myeloid or lymphoid lineages (pluripotency) [2].

Hematopoietic stem cells and progenitors can be identified by the expression of specific surface proteins that function as markers. The use of fluorescent antibodies directed against such surface markers, combined with the fluorescence activated cell sorting (FACS) technique allows the isolation of different populations of blood cells. In 1988, Spangrude et al tried to identify a minimal set of markers to define and isolate the HSC compartment [3]. Since then, different combinations have been adopted and optimized to isolate HSCs and progenitors, both in human and mouse.

Three types of HSCs have been detected in the mouse, according to the classical model of hematopoiesis [4]. Long-term HSCs (LT-HSCs) are the more undifferentiated cells, capable of reconstructing and sustaining the hematopoietic system of a lethally irradiated mouse for the rest of its lifetime; they have a low rate of proliferation and usually reside in the G<sub>0</sub> phase of the cell cycle. Conversely, short-term HSCs (ST-HSCs)

are actively proliferating cells that are able to sustain hematopoiesis for a limited period of time, namely 6-8 weeks. The third pool of HSCs is composed of committed progenitors, called multipotent progenitors (MPPs): they are actively proliferative cells that have lost self-renewal ability and give rise to the progenitors of the myeloid and the lymphoid lineages. LT-HSCs, ST-HSCs and MPPs form the LSK compartment (c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, Lin<sup>-</sup>), as they share the expression of two markers of stemness, c-Kit and Sca-1, but do not express a panel of 5 lineage specific surface markers, consisting of Gr1 (granulocytic), Mac1 (monocytic/macrophagic), TER119 (erythroid), B220 (B lymphocytic), CD3e (T lymphocytic). The three LSK populations are distinguishable by the different expression of two markers, CD34 and FLT3: LT-HSCs are defined as LSK CD34<sup>-</sup> FLT3<sup>-</sup>, ST-HSCs are LSK CD34<sup>+</sup> FLT3<sup>-</sup> while MPPs are LSK CD34<sup>+</sup> FLT3<sup>+</sup>.

Cells directly deriving from the division of MPPs form two groups of progenitors, the common lymphoid progenitors (CLPs) and the common myeloid progenitors (CMPs), responsible of the lymphoid and myeloid lineages, respectively. CLPs were identified for the first time in 1997, by Kondo et al. [5], as Lin<sup>-</sup>IL-7R<sup>+</sup>Thy-1<sup>-</sup>Sca-1<sup>lo</sup>c-Kit<sup>lo</sup>. The CLP population is composed of oligopotent cells which have lost the ability of forming myeloid cells and present a strongly decreased expression of the stemness markers, c-Kit and Sca-1. It is the first population along the hematopoietic cascade that express the receptor for IL-7, an important and necessary cytokine for the B and T differentiation.

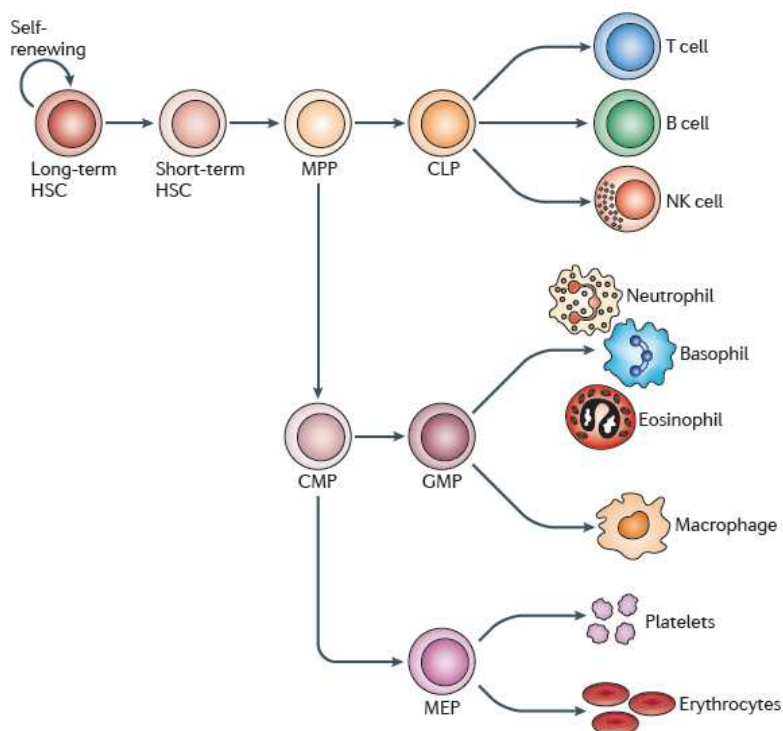
A few years later CMPs were isolated and identified as Lin<sup>-</sup> Thy1<sup>-</sup> IL7R $\alpha$ <sup>-</sup> Sca1<sup>-</sup> c-Kit<sup>+</sup> Fc $\gamma$ RI<sup>lo</sup> CD34<sup>+</sup> [6]. They give rise to more committed cells, the granulocyte and macrophage progenitors (GMP) which are defined as Lin<sup>-</sup> Thy1<sup>-</sup> IL7R $\alpha$ <sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup> Fc $\gamma$ RI<sup>hi</sup> CD34<sup>+</sup> and differentiate into granulocytes and monocytes, and the megakaryocyte and erythrocyte progenitors (MEP), which can be isolated by the



combination of Lin<sup>-</sup> Thy1<sup>-</sup> IL7R $\alpha$ <sup>-</sup> Sca-1<sup>-</sup> c-Kit<sup>+</sup> Fc $\gamma$ RI<sup>lo</sup> CD34<sup>-</sup> and give rise to megakaryocytes/platelets and red blood cells [1, 7].

In the human hematopoietic cascade, long-term and short term HSCs have not been identified, but one pool of HSCs is generally defined by a CD34/CD90 positive and CD38/Lin/CD45R $\alpha$  negative phenotype (CD34<sup>+</sup> CD38<sup>-</sup> Lin<sup>-</sup> CD90<sup>+</sup> CD45R $\alpha$ <sup>-</sup>). The MPP population is identified by the loss of CD90 expression (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>-</sup> CD90<sup>-</sup> CD45R $\alpha$ <sup>-</sup>). As in the mouse, in the human hematopoietic cascade two common progenitor populations have been identified: the CMP (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>+</sup> IL-3R $\alpha$ <sup>lo</sup> CD45R $\alpha$ <sup>-</sup>), responsible for the myeloid lineage, and the CLP (Lin<sup>-</sup> CD34<sup>+</sup> CD38<sup>+</sup> CD10<sup>+</sup>) which accounts for B and T lymphocytes and NK cells [1].

In a recent paper, new insights into the distinction between HSC and MPP have been provided suggesting that HSCs can be identified by the specific expression of CD49f, whose expression is lost in MPP [8].



**Figure 1. Hematopoietic cascade.** Hematopoiesis may be depicted as a cascade, starting with hematopoietic stem cells that gives origin to progenitors at different differentiation stages and in turn to all blood lineages. HSC = hematopoietic stem cell, MPP = multipotent progenitor, CLP = common lymphoid progenitor, NK = natural killer, CMP = common myeloid progenitors, GMP = granulocyte-macrophage progenitors, MEP = megakaryocyte-erythrocyte progenitors. From [7].

## 2. ACUTE MYELOID LEUKEMIA

### 2.1. Classification of AML

Acute myeloid leukemia (AML) is a group of clinically and molecularly heterogeneous diseases characterized by the presence of immature white blood cells (myeloblasts) in the bone marrow and peripheral blood. AML pathogenesis is associated with abnormal cell proliferation, block in differentiation and/or suppression of apoptosis. Infiltration of the bone marrow with leukemic blasts interferes with the normal production of blood cells, causing anemia, fatigue, increased risk of infections and bleeding [9].

AML can be classified according to two different systems: the FAB and WHO classifications. The French-American-British (FAB) method is based on histologic and cytogenetic parameters and distinguishes 8 main subtypes of AML based on the morphology and maturation of leukemic blasts [10, 11].

**Table 1. FAB classification of AML**

Type	FAB categories
<b>M0</b>	Undifferentiated acute myeloblastic leukemia
<b>M1</b>	Acute myeloblastic leukemia with minimal maturation
<b>M2</b>	Acute myeloblastic leukemia with maturation
<b>M3</b>	Acute promyelocytic leukemia
<b>M4</b>	Acute myelomonocytic leukemia
<b>M4eo</b>	Acute myelomonocytic leukemia with eosinophilia
<b>M5</b>	Acute monocytic leukemia
<b>M6</b>	Acute erythroid leukemia

<b>M7</b>	Acute megakaryocytic leukemia
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The FAB classification does not supply information about the prognostic perspective of the disease. Conversely, the classification of the World Health Organization (WHO) is based not only on the genetics but also on immunophenotypic, clinical and prognostic aspects, subdividing AML into 7 main categories and several subcategories.

**Table 2. WHO classification of AML**

<b>WHO categories</b>
Acute myeloid leukemia with recurrent genetic abnormalities
Acute myeloid leukemia with myelodysplasia-related changes
Therapy-related myeloid neoplasms
Acute myeloid leukemia, not otherwise specified
Myeloid sarcoma
Myeloid proliferations related to Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

**2.2. Two-hit model**

It is well accepted that many of the leukemic mutations are not able to generate AML *per se*, but at least two independent mutations (“hits”) are necessary for disease establishment.

Kelly and Gilliland described the different genetics of chronic and acute myeloid leukemia: while chronic myeloid leukemias (CMLs) are caused by constitutively activated tyrosine kinases that confer a proliferative advantage to hematopoietic

progenitors, AMLs are characterized by a cooperation between mutations in the tyrosine kinases and alterations in the differentiation process and only when the two processes, proliferation and differentiation, are altered, AML may develop [12].

Indeed, more than one function - proliferation, apoptosis, differentiation - needs to be altered to start the leukemogenic process. AML mutations are indeed classified in four groups depending on the cellular function which they are involved in [13]:

- Class I: genes involved in signal transduction. Mutations in these genes generally lead to an increase of cellular proliferation. Examples of class I genes are FLT3 and c-KIT;
- Class II: mutations affecting genes involved in differentiation, such as RUNX1, PU.1 and RAR $\alpha$ , which cause a block in cell differentiation;
- Class III: epigenetic modifiers, e.g. IDH 1/2, TET2, DNMT3A;
- Class IV: mutations in oncosuppressor genes, namely TP53 and WT1.

### **2.3. Clonal heterogeneity in AML**

AML patients harbor several mutations in their leukemic blasts: some of which are necessary for leukemia development and progression (driver mutations) while the majority are stochastic mutations due to hyper-proliferation, but confer no selective advantage to the leukemic clone (passenger mutations).

A recent advance in leukemia research includes the aim to unravel the dynamics of disease progression by understanding the timing of appearance of mutations during clonal evolution, thereby identifying which mutations are initiating events or cooperating alterations [14]. Stem/progenitor cells possess several genetic abnormalities that can be considered as passenger mutations that accumulate over time. These mutations are not able to initiate leukemogenesis. The appearance of a driver mutation affecting one of the 4 classes of genes mentioned in paragraph 2.2

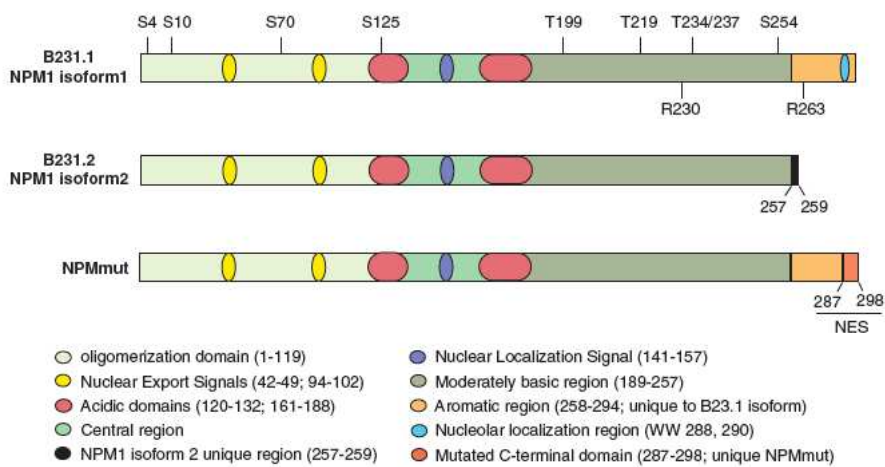
gives a proliferative advantage to the “mutated” cell that then expands into a clone. The original, founding clone possesses all the passenger mutations of the pre-leukemic cells and the initiating mutation. Subclones develop from the first and subsequent clones by acquisition of further passenger and driver mutations, increasing the genomic complexity of AML blasts at the time of diagnosis. A recent study comparing mutations found in AML blasts of 6 patients versus their normal counterpart showed that 32 out of 51 mutations were already present in non-leukemic hematopoietic stem cells [15]. Interestingly, 7 out of 13 mutations typically found in AML, such as TET2 and NPM1, are present in non-leukemic cells, which the authors define as “preleukemic” stem cells [15], prone to gaining a proliferative advantage and become leukemic stem cells.

Similar studies were performed to compare the clonal heterogeneity at the onset and at relapse of the disease. Ding and collaborators analyzed results of whole genome sequencing of samples of 8 AML patients, comparing mutations in the relapse versus the primary leukemia. They suggest two possible clonal evolution patterns in relapse: either the same initiating clone of the primary leukemia gains new mutations and evolves in the relapse clone, or a new subclone of the initiating cell emerges by gaining new mutations, thus evolving into the relapse initiating clone. Further analyses support the first hypothesis, suggesting that small subclones of the primary leukemia can bypass chemotherapy and develop into the relapse-responsible clones [16]. These studies are generating a new outlook on leukemia progression and relapse by challenging the concept of tumor clonality.

### 3. Nucleophosmin and acute myeloid leukemia

#### 3.1. NPM1 and NPMc+

Nucleophosmin (NPM1), also called B23 or numatrin, is a ubiquitously expressed phosphoprotein of the nucleoplasmin family of chaperones. NPM1 is encoded by *NPM1* gene which is formed by 12 exons and localizes in the long arm of the chromosome 5. Due to alternative splicing, two isoforms are reported: B23.1, that is more highly expressed and displays a nucleolar localization, and B23.2, localized mainly in the nucleoplasm and whose biological significance is still mostly unknown. NPM1 can shuttle between the nucleus and the cytoplasm [17], controlled by the balance between signals in the protein sequence: two nuclear export signals (NES), one nuclear localization signal (NLS) and one nucleolar localization signal (NoLS).



**Figure 2. NPM1 and NPMc+.** Cartoon representing domains and cellular localization signals of the two NPM1 isoformes, called B23.1 and B23.2. B23.1 is the most abundant within the cells, whereas B23.2 lacks a C-terminal domain. The altered C-terminal domain present in NPMc+, also called NPMmut, is depicted in orange. From [18]

NPM1 is involved in a wide range of fundamental cellular processes, among them transport of pre-ribosomal particles across the nuclear membrane and ribosome biogenesis, centrosome duplication and response to stress (reviewed in[19]). In keeping with its chaperone activity, it interacts with histones, enhancing DNA binding

and the assembly of nucleosomes [20]. NPM1 has also been reported to interact with and control the localization and stabilization of well known tumor suppressors including p53[21], Arf [22] and Fbw7 $\gamma$ [23] , suggesting its involvement in proliferative and apoptotic pathways.

NPM1 is over-expressed in a variety of cancers, including gastric, ovarian and colon carcinomas, and its overexpression is associated with an increase in cellular survival, caused by the inhibition of pro-apoptotic pathways (reviewed in [19]). Mutations of the gene as well as chromosomal translocations are characteristics of myeloid and lymphoid disorders: it has been suggested that NPM1 can help the dimerization and oligomerization process of derived oncogenic fusion proteins, namely NPM-ALK, NPM-RAR $\alpha$  and NPM-MLF1 [24].

### **3.2. NPMc+ AML**

Mutations in *NPM1* are detected in approximately 30% of AML and it is the most common genetic alteration found in the large group of AML without chromosomal translocations (normal karyotype, NK-AML) [25]. So far, more than 40 mutations have been described, the vast majority in the last exon of *NPM1*: the most frequent mutation, called mutation A, encompasses around 70% of cases and consists of the insertion (duplication) of a TCTG tetranucleotide after the position 959 of the *NPM1* coding sequence [26]. All mutations cause a frameshift in the 3' region of the gene, resulting in the loss of one or both tryptophan residues at positions 288 and 290. This modification leads to the disruption of the NLS and the formation of a de novo NES, thus altering the balance of the localization signals. Consequently, the protein encoded by the mutated *NPM1* gene is mislocalized to the cytoplasm [27-29]. The name of the mutated protein, NPMc+, and the associated leukemia, NPMc+ AML,

derive from the cytoplasmic localization of the mutated NPM1 protein in AML patients' blasts [30].

NPMc+ AML has been introduced as a provisional entity under the "Acute myeloid leukemia with recurrent genetic abnormalities" category in the 2008 version of WHO classification of hematopoietic diseases [31]. Conversely, based on the FAB classification, NPMc+ AMLs are present in all subtypes except for M3, but it is more frequent in the more mature myelomonocytic subtypes M4 and M5 [25, 32]. *NPM1* mutations are mutually exclusive with recurrent leukemic chromosomal translocations, such as AML1-ETO and PML-RAR $\alpha$  [33]. Some evidence suggests that NPMc+ is a primary mutation in the disease: it is often the only alteration found in leukemic blasts [34] and when present, other genetic alterations can be detected only in a fraction of the leukemic clone, suggesting that they may represent secondary events in leukemic progression [35].

Moreover, NPMc+ AMLs are often CD34 negative [36] and are strongly associated with the appearance of FLT3-ITD (internal tandem duplication of the *fms*-related tyrosine kinase 3 gene) [25]: FLT3-ITD+ AMLs have a poor prognosis compared to FLT3-ITD- due to a different response to chemotherapy [32, 37-39].

NPMc+ AML is characterized by a specific gene expression profile: members of homeobox genes of group A and B, as well as MEIS1 and PBX3 are overexpressed [40]. Other genes involved in the maintenance of hematopoietic stem state are positively modulated [40], suggesting that the mutation appears in an early progenitor, as supported also by the multi-lineage involvement [41]. Moreover, it is associated with a specific miRNAs profile, in part regulating Hox genes [42, 43].



### 3.3. NPMc+ in the establishment of the disease

The mechanism of action of NPMc+ in the establishment and/or progression of the disease is still not completely understood. However, the balance between the amounts of wild-type (wt) and mutant proteins and protein dosage seem to play an important role.

As reported by Grisendi et al. [44], *NPM1* haploinsufficiency leads to an increased genomic instability, suggesting a decrease in NPM1 function in centrosome duplication during mitosis. Moreover, the authors showed that lack of one allele is sufficient *per se* for erythroid and megakaryocytic dysplasia, features of human myeloproliferative neoplasms [44]. In AML patients, the situation is less striking: correct centrosome duplication seems to be maintained, as suggested by the strong association of NPMc+ AML with normal karyotype. Nonetheless, the function may be partially impaired, as supported by the low number of numerical chromosome alterations found in patients.

Decrease of the wt function and the concomitant presence of the mutant form can interfere with the correct function of pathways controlling DNA damage. NPM1 is indeed able to interact and control the localization and stabilization of regulators of genome stability, namely p53, HDM2 (and its murine counterpart MDM2) and ARF. ARF is generally sequestered in the nucleolus via NPM1 and is released upon stress stimuli in order to stabilize p53 in the nucleoplasm, leading to activation of pathways of apoptosis, cell cycle arrest and/or senescence. It has been proposed that part of the leukemogenic potential of NPMc+ resides in the maintenance of its interactions with partners of wt NPM1, thereby delocalizing partner proteins to the cytoplasm and interfering with their function. In the case of ARF, NPMc+ delocalizes the protein to the cytoplasm, increasing its instability and its degradation and thus impairing p53 functions [45]. The expression of NPMc+ also leads to the cytoplasmic delocalization

of Fbw7 $\gamma$ , a regulator of c-Myc that interacts with NPM1: Fbw7 $\gamma$  is no longer able to contribute to the ubiquitination and degradation of c-Myc, whose cellular level increases, promoting cell proliferation [23].

NPMc+ might have a dominant negative effect on its wt counterpart: the mutant form can interact with the wt, leading to a decrease in the nuclear pool of NPM1 and limiting the amount of protein available for the completion of its functions [27, 29].

The relevance of a fine control on NPM1 dosage was demonstrated by *in vitro* experiments: in fact, overexpression of NPM1 enhances cell growth and inhibits apoptosis [19]. Maggi and coworkers demonstrated that NPM1 levels correlate with and control the rate of protein synthesis: decreased levels of NPM1 diminished the nuclear export of both the 40S and 60S ribosomal subunits while a modest increase of NPM1 enhanced the export of newly synthesized rRNAs; the authors concluded that NPM1 itself is rate limiting in this process [46].

NPM1 can directly and indirectly regulate transcription. It contributes to the control of chromatin accessibility due to its activity as a histone chaperone and its role in nucleosome assembly. It recruits histone acetyl transferases (HATs) and deacetylases (HDACs) to chromatin, thus regulating the acetylation state of DNA [47], and it acts as modulator of specific transcription factors like YY1 and NF- $\kappa$ B [48, 49]. Moreover, NPM1 interacts with hexamethylene bis-acetamide-inducible protein (HEXIM1), an inhibitor of the elongation of Polymerase II (PolII) transcripts: NPMc+ delocalizes HEXIM1 to the cytoplasm, with an enhancement of PolII transcription [50].

Other studies describe diverse functions of NPM1 and NPMc+. For example, Sagawa and colleagues confirmed that NPM1 is deposited on mRNA and influences mRNA 3' end processing and nuclear export [51]. In 2013, Noguera et al. identified a novel interactor of NPM1: both the wt and the mutant proteins can bind to herpes virus-associated ubiquitin specific protease (HAUSP) [52]. In the cytoplasm, HAUSP

regulates the deubiquitination of PTEN, helping its entry into the nucleus. The binding of NPM1 and NPMc+ to HAUSP inhibits its effect on PTEN, which is degraded through the proteasome. The authors conclude that this mechanism of nuclear PTEN depletion can contribute to survival and proliferation of leukemic cells [52].

In summary, NPM1 is a pleiotropic protein with multiple functions in cell homeostasis and its mutant NPMc+ may therefore be capable of interfering with multiple cellular processes.

### **3.4. NPMc+ animal models**

Several animal models have been generated to elucidate the molecular mechanism underlying the development of AML bearing NPM1 mutations.

The ablation of the *Npm1* gene in mice results in embryonic lethality between E11.5 and E12.5: *Npm1*<sup>-/-</sup> embryos are smaller in size compared to wt and show developmental defects, including deficient anterior brain organogenesis and hematopoietic defects [44, 53]. In particular, they have a reduced number of hematopoietic precursors and a decreased number of blood islands in the yolk sac; the differentiation ability of hematopoietic precursors is also impaired [44]. A milder phenotype is showed by a homozygous hypomorphic mutant, *Npm1*<sup>hy/hy</sup>, confirming the important role of NPM1 in development of brain and blood [44]. Heterozygous *Npm1*<sup>+/-</sup> mice are viable and show features of human myelodysplastic syndrome (MDS), such as increased mean corpuscular volume and abnormal platelet count [44]. Cheng and coworkers described the first mouse model carrying NPMc+ in which NPMc+ expression was driven by a human myeloid promoter (MRP8) and led to features of myeloproliferation in bone marrow and spleen, as shown by enhancement of the mature granulocyte/monocyte (Mac1<sup>+</sup>, Gr1<sup>+</sup> cells) compartment [54]. A myeloproliferative phenotype was reported also in zebrafish: knockdown of

endogenous *npm1a* in zebrafish embryos resulted in a reduction in the number of myeloid cells that was specifically rescued by human NPM1, while the expression of NPMc+ in zebrafish embryos led to expansion of primitive and definitive hematopoietic cells [55].

In 2011, Vassiliou and collaborators described the leukemic phenotype of a conditional knock-in mouse model carrying a humanized mutation of NPM1: the region surrounding the TCTG mutation in the exon12 of *NPMc+* was inserted in the corresponding region of exon 11 of *Npm1* [56]. The peculiarity of this model is that the mutated allele is controlled by the endogenous promoter of *Npm1*, maintaining the balance of mutated versus wt alleles. These mice showed enhancement of *Hox* gene expression, a feature shared with NPMc+ AML in humans [40], and myeloproliferation followed by development of late-onset AML, suggesting that NPMc+ is not sufficient *per se* for leukemic transformation but requires additional mutations [56]. Taking advantage of the Sleeping Beauty system, the authors identified known and not yet described cooperating mutations, such as for example, mutations in *Nf1* and *Nup98* genes [56].

One of the known cooperating mutation of NPMc+ in leukemia establishment is FLT3-ITD. Mice carrying both the NPMc+ and FLT3-ITD mutations developed AML with short latency as reported in two independent studies [57, 58].

The interaction of hematopoietic stem cells (HSC) with their niche also appears to be important for the myeloproliferative effect of NPMc+. A recent knock-in mouse model that mimics the human mutation showed the downregulation of CXCR4/CXCL12 related genes, suggesting that a modification in the HSC/niche interaction could be part of the mechanism of action of NPMc+[59].

## 4. WNT SIGNALLING

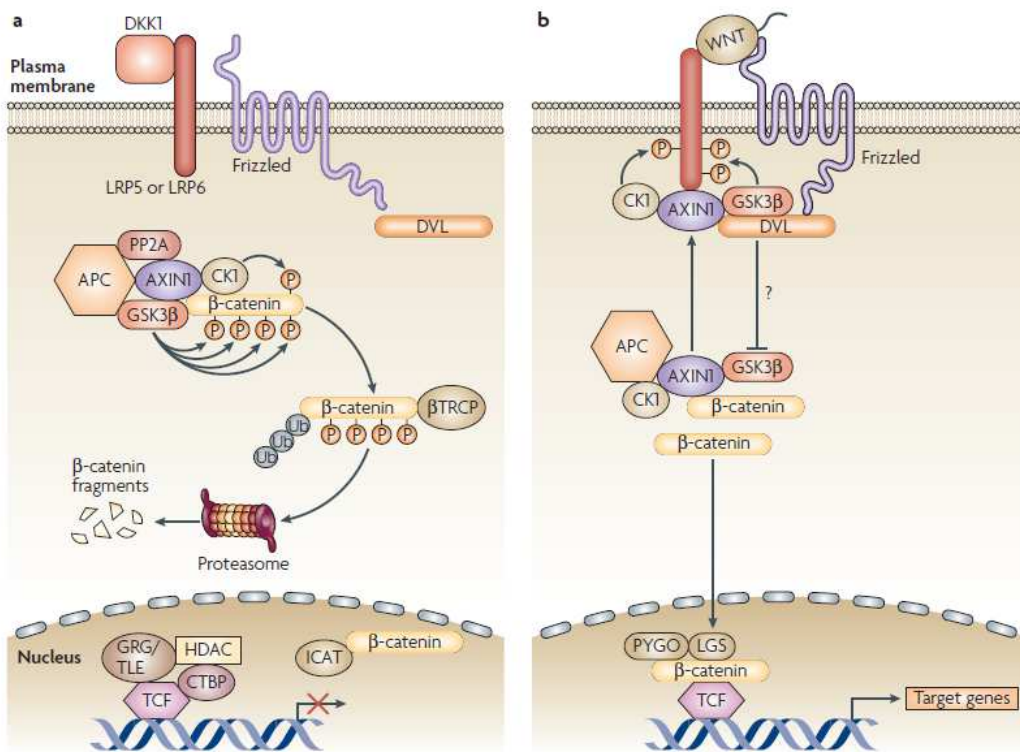
### 4.1. Canonical and non-canonical pathways

A common feature of many subtypes of AML is the deregulation of Wnt signaling, which has a pivotal role in the maintenance of HSCs and in differentiation of blood cells [60].

The Wnt pathway is activated by lipid-modified extracellular molecules, the Wnt ligands, that bind to receptors at the membrane of responding cells, triggering modification in the cellular structure and gene/protein expression profile. 19 genes in murine and human genomes encode different Wnt ligands that bind to members of a family of seven-pass transmembrane receptors called Frizzled, which transmit the signal inside the cell.

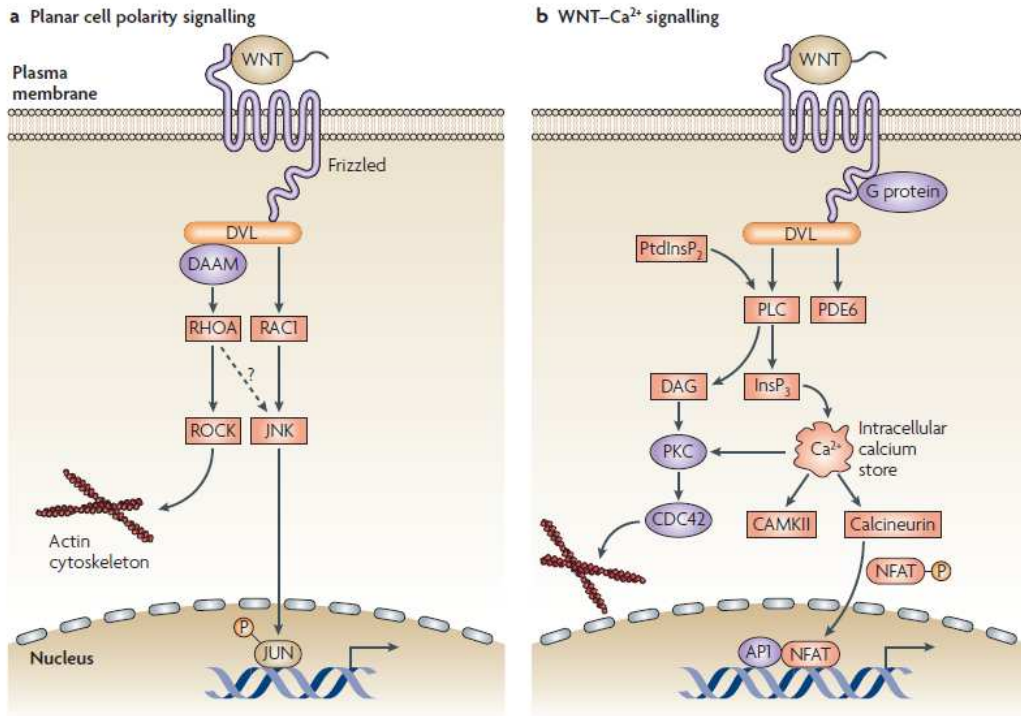
In the absence of Wnt ligands,  $\beta$ -catenin, the main effector of the so-called canonical pathway, is sequestered in a multiprotein cytoplasmic complex (“destruction complex”) which includes AXIN, Adenomatous Polyposis Coli (APC), glycogen synthase kinase 3 beta (GSK3 $\beta$ ) and casein kinase 1 (CK1).  $\beta$ -catenin is phosphorylated by GSK3 $\beta$  and CK1, ubiquitinated by beta-transducing repeat-containing protein ( $\beta$ TRCP) and then degraded in the proteasome [60-62].

The pathway becomes active when the Wnt ligand binds to the receptor. This binding causes activation of the receptor and phosphorylation of the co-activator LRP5/6 by GSK3 $\beta$  and CK1: together with the scaffold protein DVL (Dishevelled), they recruit AXIN to the membrane, resulting in the stabilization of  $\beta$ -catenin and its accumulation in the cytoplasm.  $\beta$ -catenin can then enter the nucleus and bind to transcriptional co-activators of the TCF/LEF1 family to direct the expression of target genes, such as CyclinD1, AXIN and TCF [63].



**Figure 3. Canonical Wnt signaling.** Not active (a) and active (b) canonical Wnt signaling are depicted in these two panels. When the pathway is not active,  $\beta$ -catenin is phosphorylated within the destruction complex formed by APC, GSK3 $\beta$ , CK1 $\alpha$  and other scaffold proteins. Phosphorylated and ubiquitinated  $\beta$ -catenin is degraded through proteasome. The activation of the pathway avoids the formation of the destruction complex,  $\beta$ -catenin accumulates and activates the expression of target genes. DKK1 = Dickkopf homologue 1, LRP5/6 = LDL-receptor-related protein 5/6, DVL = mammalian homologue of *Drosophila* Dishevelled, APC = adenomatous polyposis coli, PP2A = protein phosphatase 2A, GSK3 $\beta$  = glycogen synthase kinase 3 $\beta$ , CK1 = casein kinase 1, AXIN1 = axis inhibition protein 1,  $\beta$ -TRCP =  $\beta$ -transducin-repeat-containing protein, GRG/TLE = Groucho/transducin-like enhancer, HDAC = histone deacetylase, CTBP = C-terminal binding protein, TCF = T-cell factor, ICAT = cell autonomous inhibitor of  $\beta$ -catenin and TCF, PYGO = Pygopus, LGS = legless. From [63].

Different Wnt ligands such as Wnt5a and Wnt4 are able to activate other signaling pathways, defined as non-canonical. The two better characterized non-canonical pathways are the Wnt - Ca<sup>2+</sup> and the planar cell polarity (PCP) pathways. In the first, the interaction between the Wnt ligand and the receptor leads to an increase of intracellular calcium concentration with the subsequent activation of calcium-sensitive enzymes, such as PKC and CaMKII. In the PCP pathway, ligand-receptor binding results in the activation of RHOA and RAC leading to the activation of JNK kinase and cytoskeleton rearrangement [64].



**Figure 4. Non canonical Wnt signaling.** Wnt ligands are able to activate pathway that do not have  $\beta$ -catenin as principal effector. Planar cell polarity pathway (a) leads to the activation of JNK (Jun N-terminal kinase) and the expression of JUN target genes and to alteration in the cellular cytoskeleton. Wnt -  $\text{Ca}^{2+}$  signaling (b) is based on the increase of intracellular concentration of calcium and the activation of  $\text{Ca}^{2+}$  - sensible enzymes, namely CAMKII (calcium calmodulin mediated kinase II) and PKC (protein kinase C). DVL = mammalian homologue of *Drosophila* Dishevelled, DAAM = Dishevelled-associated activator of morphogenesis, RHOA = RAS homologue gene-family member A, RAC1 = ras-related C3 botulinum toxin substrate 1, ROCK = RHO-associated coiled-coil-containing protein kinase 1,  $\text{PtdInsP}_2$  = phosphatidylinositol-4,5-bisphosphate, PLC = phospholipase C, PDE6 = phosphodiesterase 6, DAG = diacylglycerol, CDC42 = cell-division cycle 42,  $\text{InsP}_3$  = inositol trisphosphate, NFAT = nuclear factor of activated T cells, AP1 = activator protein 1. From [63].

#### 4.2. Inhibition of the canonical Wnt pathway

The canonical Wnt pathway can be inhibited by physiological molecules and chemical compounds. Dickkopf (DKK) is a family of secreted proteins, rich in cysteines, that recognize and bind to the LRP5/6 co-receptor, preventing its interaction with Frizzled and inhibiting the activation of the receptor itself [65]. Four members of the family are encoded in the human genome (three in mouse), called *DKK1-4*, and elicit different effects on Wnt signaling, probably through the interaction with another receptor called Kremen [66]. The first member of the family, DKK1 is the orthologue of the *Xenopus* *dkk1*, whose effect on the canonical pathway and on the vertebrate development has been extensively studied. In 1998, Glinka and

colleagues described dkk1 as a potent Wnt inhibitor and a head inducer, showing that its over-expression lead to the formation of a secondary head [67]. Dkk1 is necessary for head formation also in mammals, as shown by Mukhopadhyay et al. [68] in mice.

DKKs are not the only inhibitors of the pathway. Wnt ligands can bind to secreted forms of the frizzled molecules, called Secreted Frizzled Related Proteins (SFRPs), avoiding the interaction with the transmembrane receptor and thus attenuating the signal [69]. Other inhibitors are Wnt inhibitory factor (WIF), which binds to Wnt ligands , and ICAT (inhibitor of  $\beta$ -catenin and TCF-4) that interacts with  $\beta$ -catenin preventing its interaction to TCF/LEF complex [70].

A large series of compounds that act as Wnt signaling inhibitors has been discovered or developed. First, antibodies against the Wnt ligands or the Frizzled receptors can easily block or reduce the signal, but other components of the pathway can also be targeted [70]. NSC668036 is a chemical compound that recognizes and binds to the PDZ domain of DVL, inhibiting its role as scaffold protein in the recruitment of AXIN and APC to the membrane [70].  $\beta$ -catenin is also a target: non-steroidal anti-inflammatory drugs, such as indomethacin and sulindac derivates, can indirectly decrease the level of total  $\beta$ -catenin, modulating the signal [71, 72]. Finally, two other groups of compounds can inhibit the pathway: the inhibitors of Wnt response (IWR), which interact with and stabilize Axin and therefore the entire destruction complex, and the inhibitors of Wnt production (IWP), which interact with Porcupine, a protein involved in the secretion of Wnt ligand, thereby decreasing their release in the extracellular environment [70].

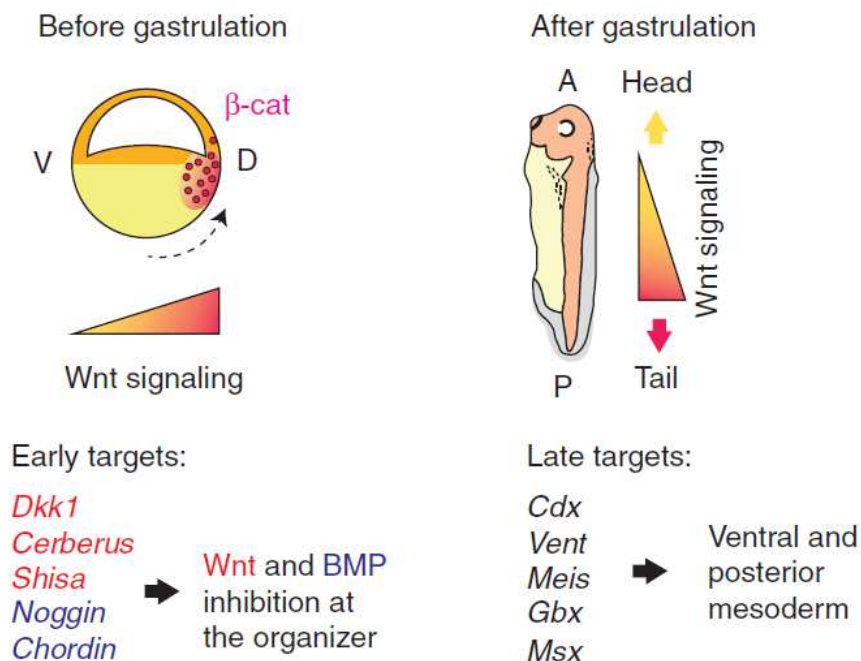


### 4.3. Wnt in development

Wnt pathways are involved in many developmental processes. Wnt signaling is indeed fundamental for the specification of the dorso-ventral and antero-posterior axis in the developing embryo.

Early Wnt signaling is important for dorso-ventral axis formation: experiments conducted in *Xenopus* and zebrafish showed that the accumulation of  $\beta$ -catenin in the dorsal nuclei of the fertilized eggs is fundamental for the formation of the organizer, a region that dictates the future dorso-ventral patterning of the embryo [73, 74]. Moreover, Wnt molecules in the ventral region of the embryo at gastrulation indirectly limit expression of organizer specific genes, like *chordin* [74, 75].

Evidence that the overexpression of *Wnt8* through two different techniques (mRNA or plasmid injection) gives rise to different phenotypes (secondary axis formation in the first case, posteriorization of the embryo in the latter) suggests that the involvement of Wnt signaling in development is stage-specific [73].



**Figure 5. Effect of Wnt pathway during early development.** Activation of Wnt signaling before gastrulation plays an important role in the determination of the dorso-ventral axis, with Wnt pathway components that are more present in the dorsal region of the embryo, together with BMP pathway. After gastrulation, Wnt signaling dictates the antero-posterior fate: Wnt pathway is more active in the

posterior part of the embryos where it activates late target genes which are necessary for ventral and posterior mesoderm formation.  $\beta$ -cat =  $\beta$ -catenin, BMP = bone morphogenetic pathway, Cdx = caudal type homeobox, Vent = ventral expressed homeobox, Gbx = gastrulation brain homeobox, Msx = muscle segment homeobox. From [73].

Formation of the antero-posterior axis is determined by two gradients of molecules in a later stage of development, after gastrulation: a group of Wnt ligands, activators of the pathway, are expressed in the posterior part of the embryos while an opposite gradient of Wnt inhibitors, like Frzb and Dkks, springs from the region of the head and counteracts the posteriorizing effect of Wnts [73, 74]. The role of Wnt in antero-posterior specification has been proved also in mammals by the phenotype of knockout mice lacking genes involved in the Wnt pathway [73, 74].

Non-canonical Wnt signaling, in particular the PCP pathway, is involved in an important step in the early stages of development. Cellular remodeling and movement are fundamental processes for the correct formation of the embryos during vertebrate development, and their deregulation leads to mislocalization of future adult tissues: groups of cells belonging to the three germ layers are localized correctly by movements of convergence and extension (CE movements). Wnt11 and Wnt5a are necessary for the correct formation of the head because of their involvement in CE processes [76, 77].

#### **4.4. Wnt in hematopoiesis**

The first evidence of Wnt signaling activation in hematopoiesis emerges from a study in 1997, which demonstrated that Wnt ligands were expressed in murine fetal liver [78]. Further studies revealed that Wnt ligands and receptors are expressed in the murine yolk sac, aorta-gonad-mesonephros and fetal liver, sites of murine primitive hematopoiesis [79]. Gain- and loss-of-function experiments confirmed the importance of Wnt signaling in mammalian primitive hematopoiesis. First, treatment

with Wnt10b increased proliferation of murine fetal liver hematopoietic progenitors *in vitro* [78], while Wnt3a<sup>-/-</sup> mice showed a strong reduction of hematopoietic progenitors in fetal liver and embryonic lethality [80]. Recently, Clements and collaborators demonstrated that the expression of Wnt16 is required for the beginning of definitive hematopoiesis [81].

Wnts are indeed expressed in HSCs and their microenvironment: Van den Berg and coworkers showed that three Wnt ligands, Wnt2, Wnt5a and Wnt10b, are expressed in human bone marrow cells and, in particular Wnt5a is expressed in a pool of HSCs and progenitors [82], while Wnt3a, Wnt5a and Wnt10b are expressed in murine bone marrow [83]. In a recent paper, Luis and collaborators took advantage of the Axin2<sup>LacZ</sup> reporter mice to analyze canonical Wnt signaling activation in different hematopoietic populations: this model allows the identification of cells expressing AXIN2, a well known target of the  $\beta$ -catenin dependent pathway. They showed that Wnt signaling presents different levels of activation in different subsets: Wnt is mildly activated in HSCs and progenitors and strongly activated in thymocytes, while it is reduced or absent in mature myeloid cells and along the B lineage [84].

To better analyze the role of Wnt signaling in hematopoiesis, several gain- and loss-of-function experiments have been conducted. One of the first studies revealed that transduction of a constitutively active  $\beta$ -catenin in Bcl2 expressing hematopoietic stem/progenitor cells increased both proliferation *in vitro* and repopulation capacity in lethally irradiated mice [85]. More recently, activation of Wnt signaling by administration of a GSK3 $\beta$  inhibitor has been reported to enhance engraftment of both murine and human HSCs [86, 87]. Huang and coworkers described a dual effect of GSK3 $\beta$  inhibition, an expansion of HSCs and the concomitant decrease of their reconstitution ability [88]. Similar results were obtained in transgenic mice which expressed a stabilized form of  $\beta$ -catenin: transient expansion of HSCs, differentiation

block and defects in reconstitution [89, 90]. Conversely, the combination of pro-survival pathways (Akt activation through PTEN deletion) and Wnt signaling activation enhances HSCs self-renewal and reconstitution capability [91]. Moreover, another model, the *Apc*<sup>Min/+</sup> mouse which carries a mutated form of APC, showed enhanced repopulation ability of HSCs [92].

Conflicting results were also obtained by loss-of-functions experiments. For example, conditional deletion of  $\beta$ -catenin using a Vav-Cre transgene did not impair the formation of HSCs but affected their reconstitution ability [93] whereas using an IFN-inducible Mx-Cre transgene,  $\beta$ -catenin deletion did not affect either the self-renewal or the reconstitution ability of HSCs [94]. Inhibition of Wnt signaling was achieved also by overexpression of DKK1 in the osteoblastic stem cell niche by Fleming and collaborators, showing a decrease in the self-renewal capability of HSCs [95].

The recent paper by Luis and colleagues demonstrated that different levels of Wnt activation have specific effects at different steps of the hematopoietic cascade. Combining three different alleles of *Apc*, the authors obtained five Wnt signaling levels *in vivo* [84]. Low levels of Wnt activation drive the maintenance of self-renewal and reconstitution capacities of HSCs, as shown by competitive limiting dilution transplantation assay, but these properties of HSCs are inhibited by intermediate and high levels of Wnt activation. An intermediate level of Wnt activation enhances myeloid differentiation, as shown by the increased number of granulocyte/macrophage (GM) and macrophage (M) colony-forming units (CFU) in methylcellulose assay and the expansion of the GMP compartment, without affecting the number of differentiated myeloid cells. Conversely, the highest level of Wnt activation leads to a strong reduction in the number of colonies, suggesting that when the signaling is too high, the hematopoietic process is impaired [84]. This new model can explain the conflicting phenotypes that resulted from different strategies in gain-

and loss-of-function experiments; for example, the expression of a constitutively active form of  $\beta$ -catenin can induce a high activation of Wnt pathway that leads to exhaustion of HSC pool [89, 90], while in the conditional  $\beta$ -catenin knock-out model, which does not show effects on HSC behavior [94, 96, 97], a residual amount of protein (70% and 25%, [60]) may be sufficient to sustain HSC self-renewal and repopulation ability.

#### **4.5. Wnt pathway in acute myeloid leukemia**

Wnt signaling is involved not only in physiological steps of hematopoiesis but its alteration is part of the leukemogenic process.

Wnt signaling can be altered by epigenetic modifications: alteration in methylation status of Wnt inhibitors, namely *sFRPs* and *Dkks* genes, was detected in AML cell lines and patients, suggesting an upregulation of the Wnt pathway in AML [98, 99].

In recent years, correlation between leukemia-associated lesions and alteration of Wnt signaling has been assessed. Müller-Tidow and collaborators analyzed the effect of the expression of three leukemia-associated fusion proteins, AML1-ETO, PML-RAR $\alpha$  and PLZF-RAR $\alpha$  [100] and demonstrated that the fusion proteins induce expression of  $\gamma$ -catenin, a homologue of  $\beta$ -catenin.  $\gamma$ -catenin in turn activates TCF and LEF transcriptional activity and modulates expression of Wnt target genes, namely c-Myc and CyclinD1 [100]. Moreover, inhibition of the pathway through the expression of a dominant negative form of TCF4 decreases cell growth in AML1-ETO expressing Kasumi-1 cell line [100]. Another group confirmed the ability of the AML associated fusion proteins to induce the expression of  $\gamma$ -catenin by directly activating its promoter and in turn  $\gamma$ -catenin can increase the replating efficiency of HSCs [101].

Wang and collaborators demonstrated that a leukemic model expressing MLL-AF9 displays an increase of unphosphorylated, active  $\beta$ -catenin in GMPs; they also showed

that expression of a constitutively active form of  $\beta$ -catenin in a leukemic mouse model driven by the combination of HoxA9 and Meis1 contributes to the establishment of AML in recipient mice [102]. Interestingly, deletion of  $\beta$ -catenin decreased self-renewal of leukemia initiating cells and impaired leukemia development in transplanted mice [102].

Other studies revealed that canonical Wnt signaling is involved also in the development of mixed-lineage leukemia (MLL) leukemic stem cells:  $\beta$ -catenin knockdown decreased proliferation in a MLL human cell line and the frequency of CFU blasts in MLL patients [103]. The authors also demonstrated that resistance to GSK3 $\beta$  inhibitors of MLL leukemic stem cells depends on  $\beta$ -catenin [103].

## **5. Zebrafish as a tool for hematopoiesis and leukemia**

### **5.1. Overview**

Zebrafish has become a powerful model in recent years thanks to its interesting features: the large number of eggs, the external fecundation and the transparency of the embryo. It has a short life cycle: in 24-36 hours, precursors of major organs are formed [104] and it becomes adult in three months.

Several techniques of forward and reverse genetics can be applied to easily modify the zebrafish genome. Large scale mutation screening based on chemical mutagens, like N-ethyl-N-nitrosourea (ENU), or modified retroviruses allowed the discovery of genes involved in hematopoiesis: an example is the *kugelig* (*kgg*) mutant that presents a mutation in *cdx4*, a gene involved in the regulation of Hox genes, and shows severe anemia and a shortened tail [105].

Genes can be targeted by the use of morpholino or by the ZFNs (zinc finger nucleases) technique. Morpholinos are small oligonucleotides designed to recognize and bind to

the AUG region or splicing boundaries of defined mRNAs: the first inhibits the formation of the corresponding protein avoiding the interaction of the ribosome machinery with AUG, the latter interferes with splicing and leads to the formation of shorter mRNAs and truncated proteins. Morpholinos are generally used to study the effect of the knockdown of the gene of interest in early embryogenesis: they are injected into one-cell stage embryos and their effect is maintained for up to 5 days, by which degradation and dilution make them insufficient to maintain the knockdown. Before the introduction of the ZFNs technique, reverse genetics was based on chemical or retroviral mutagenesis followed by screening of mutations in the gene of interest, very similar to the forward genetic approach. Nowadays, the use of modified zinc finger nucleases enables cutting of double strand DNA within a gene of interest and knocking out the gene after imperfect DNA repair [106].

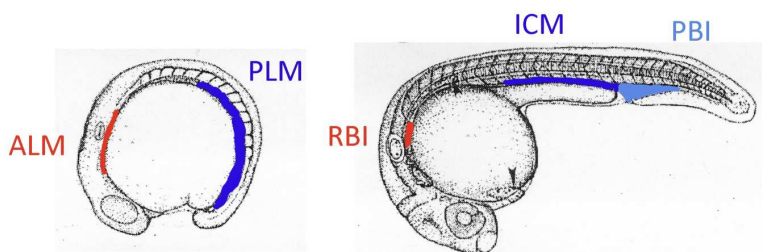
Zebrafish has been used as a model to study hematopoiesis and leukemia: zebrafish hematopoiesis (described in paragraph 5.2) is similar to the mammalian hematopoietic cascade and the accessibility of the embryos from the early stages of development allows a detailed analysis of the formation of the embryonic hematopoietic system. Moreover, the easy manipulation of the embryo, the existence of transgenic and mutant strains and the short life cycle permit faster readouts when compared to the mouse system.

Two examples of human fusion proteins that have been studied in zebrafish are AML1-ETO [107] and NUP98-HOXA9 [108]. AML1-ETO expression leads to defects in hematopoiesis and blood circulation: cytological and molecular studies revealed an accumulation of immature cells resembling leukemic blasts, and alterations in gene expression parallel to those detected in human AML1-ETO derived AML [107]. AML1-ETO was used to screen for new chemical-modifiers for the effect of the oncoprotein on hematopoiesis, revealing the involvement of COX-2- and  $\beta$ -catenin-dependent

pathways in its leukemogenic mechanism [109]. These studies demonstrate that the generation of leukemia models in zebrafish helps to uncover the molecular mechanism of the disease.

## 5.2. Zebrafish hematopoiesis

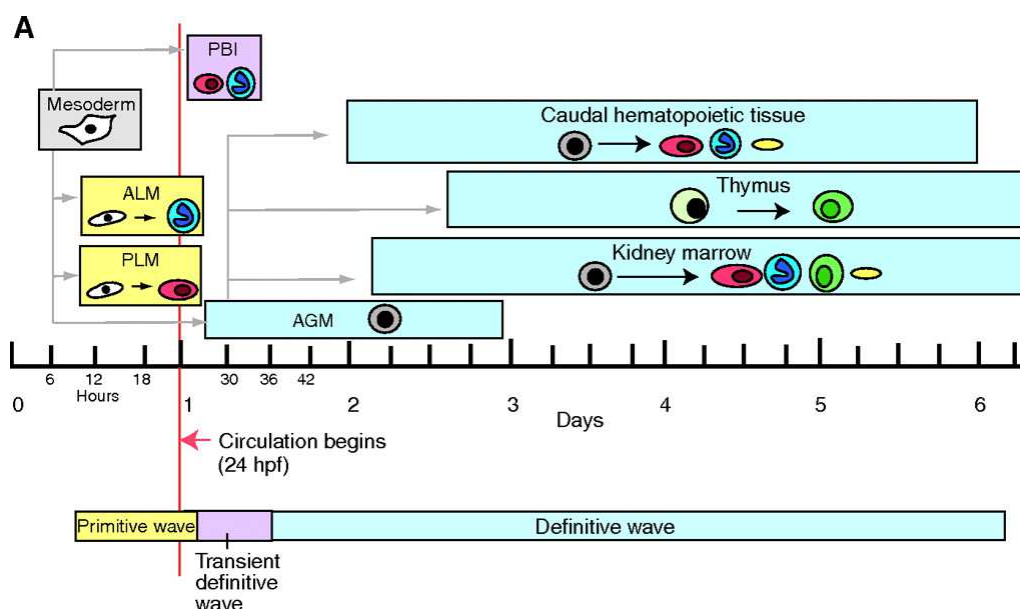
Similarly to what occurs in mammals, zebrafish hematopoiesis can be divided into distinct waves. Primitive hematopoiesis accounts for the formation of myeloid and erythroid precursor cells in the early stages of the embryo, and takes place in two locations, the anterior lateral mesoderm (ALM) and posterior lateral mesoderm (PLM), which later in development give origin to the rostral blood island (RBI) and the intermediate cell mass (ICM), respectively. A later step of primitive hematopoiesis (“intermediate hematopoiesis”) takes place in a tissue located in the posterior region of the embryo, the posterior blood island (PBI), where a group of erythromyeloid committed progenitors arises. These cells are able to differentiate into both erythrocytes and myeloid cells. Definitive hematopoiesis is responsible for the formation of all lineages, myeloid and lymphoid, present in the adult fish [110].



**Figure 6. Areas of primitive hematopoiesis in zebrafish embryo.** Primitive hematopoiesis occurs in different regions along the embryo’s body. At 10 hpf expression of *tal1*, an hematopoietic/endothelial markers appears in the anterior lateral mesoderm (ALM, depicted in red) and posterior lateral mesoderm (PLM, depicted in blue). The ALM develops in the rostral blood island (RBI) while the PLM gives rise to the intermediate cell mass (ICM). A transient waves of blood cells production occurs in the posterior blood island (PBI) at 24 hpf. Pictures modified from [111]



Primitive hematopoiesis begins at 10 hours post fertilization (hpf) when groups of cells forming two stripes in the ALM and in PLM of the embryo start to express *tal1* (also called *scf*). Two major hypothesis have been proposed for the identity if these *tal1*<sup>+</sup> cells: they could be either hemangioblasts, namely precursors of both hematopoietic stem cells and endothelial progenitors, suggesting a common origin for blood cells and vessels, or parallel populations of angioblasts and primitive hematopoietic progenitors [112]. In any case, hematopoietic precursors in ALM and PLM start to express a panel of markers, which are mostly transcription factors orchestrating hematopoiesis, like *tal1*, *lmo2* and *gata2* [112, 113].



**Figure 7. Primitive and definitive zebrafish hematopoiesis.** Zebrafish hematopoiesis may be divided in three distinct waves. Primitive hematopoiesis starts at about 10 hpf (hours post fertilization) in two areas, the ALM (anterior lateral mesoderm) and the PLM (posterior lateral mesoderm); it gives origin to myeloid (depicted in blue) and erythroid cells (depicted in red). A transient phase of both myeloid and erythroid cells production occurs later in PBI (posterior blood island) area. Definitive hematopoiesis starts at 24 hpf in the AGM (aorta gonad-mesonephros) where hematopoietic progenitors expressing *c-myb* and *runx1* appear. Hematopoietic progenitors (depicted in grey) then migrate towards definitive hematopoietic sites, thymus nad kidney marrow, after a transient phase of blood cells production in the caudal hematopoietic tissue. The definitive hematopoiesis accounts for the formation of all the zebrafish blood lineages: myeloid (blue) and erythroid (red) cells, lymphocytes (green), thrombocytes (yellow). Modified from [114]

At the stage of five somites, approximately 12 hpf, a subpopulation of cells in the PLM starts to express *gata1*, a transcription factor governing erythroid differentiation.

Cells begin differentiating in proerythroblasts and enter the blood stream at around 24 hpf. Circulating cells can be easily identified by the expression of *alas2*,  $\alpha$  and  $\beta$  hemoglobin chain genes [112, 113].

At early stages of development a small population of cells in ALM starts to express *pu.1* and to differentiate in myeloid cells. These myeloid precursors give rise to macrophages that migrate rostrally and spread on the yolk sac and along the embryo's body. *pu.1* is expressed also in the ICM and the PBI where its function in myeloid differentiation is counteracted by *gata1*. The reciprocal inhibition of the two transcription factors regulates the fate of cells in these two regions: in ICM *gata1* is predominant and only a small subpopulation differentiates into neutrophils, while in the PBI a majority of myeloid cells is generated [110].

Definitive hematopoiesis starts at about 24 hpf when cells from the ventral wall of the dorsal aorta start to express *c-myb* and *runx1*, markers of definitive hematopoietic progenitors. The endothelium of the dorsal aorta corresponds to the aorta gonad-mesonephros (AGM) region in the mouse embryo as the first site of adult HSCs formation. In zebrafish, HSCs emerge directly from the aortic floor through a *runx1*-depending process that is called endothelial-hematopoietic transition (EHT). EHT involves the bending and exiting of single endothelial cells into the sub-aortic space, transforming into hematopoietic cells [115, 116]. Around 48 hpf, *c-myb<sup>+</sup> runx1<sup>+</sup>* cells move towards the caudal hematopoietic tissue (CHT) in the posterior region of the embryo [117, 118]. Later, starting from 3-4 days post fertilization (dpf), hematopoietic progenitors are present in the thymus where they give rise to T lymphocytes, and in the pronephros. In adults, hematopoiesis takes place in the kidney, where HSCs are intercalated among the renal tubules, giving rise to erythrocytes (from 5 dpf), which differently to the mammals maintain the nucleus, B lymphocytes (from 19 dpf), granulocytes and monocytes (from 7 dpf) [112, 113].





## AIM OF THE PROJECT

The aims of this project were:

- a. to analyze the effect of both NPM1 and NPMc+ on zebrafish development and primitive hematopoiesis;
- b. to recapitulate the effects of NPMc+ in a mammalian in vitro system, in an attempt to dissect the molecular mechanism underlying the NPMc+ derived Wnt modulation.

To fulfill the first aim, we focused our attention on processes of zebrafish development that are known to be controlled by Wnt signaling. In particular, we analyzed the maintenance of correct convergence and extension movements at early developmental stages to study the effect of NPMc+ on non canonical Wnt signaling, while canonical Wnt signaling modulation was studied through the expression of *dkk1b*, a target of maternal signaling which is able to inhibit the zygotic Wnt pathway. Moreover, we took advantage of the TOP:GFP transgenic strain to monitor the activation of the canonical Wnt pathway in more developed embryos. Again, given the involvement of NPMc+ in leukemia, we analyzed the expression of hematopoietic markers (namely *gata2*, *lmo2* and *tal1* for hematopoietic precursors, *gata1* for the erythroid lineage, *pu.1* for myeloid precursors, *l-plastin* and *mpx* for mature myeloid cells).

In the second part of the project, we characterized a retrovirally infected immortalized murine hematopoietic stem/progenitor cell line that expresses NPMc+. NPMc+ cells were studied for their proliferation and myeloid differentiation capacity, expression level of Wnt target genes and response to Wnt activation and inhibition.

Finally, we analyzed activation of Wnt signaling in AML patients' blasts, through the analysis of expression of Wnt target genes and components of the canonical Wnt pathway.

## **MATERIALS AND METHODS**





## **Zebrafish strains and maintenance**

Tübingen wild-type and transgenic Tg(*gata1:dsRED*)[119], Tg(*mpx:GFP*)[120], Tg(*TOP:GFP*)[121] zebrafish strains were maintained and bred according to standard procedures [122].

## **Antisense morpholino oligonucleotides and RNA injection**

Antisense morpholino oligonucleotide was designed (GeneTools, LLC) against the exon2-intron2 splice site of *npm1a* (Splicing Block MO, SBmo). (5'-*CAGTTCACAACCTGTAAAACATAAAA*-3').

Zebrafish *dkk1b*, human *NPM1* and *NPMc+* cDNAs were cloned into *pCS2+* plasmid. 10 µg of each construct were digested with *NotI*, purified with PCR purification kit (QIAGEN) and used as template for and *in vitro* transcription using the *mMessage mMachin*e SP6 kit (Ambion) to generate capped RNAs. Zebrafish embryos were microinjected at 1 cell stage into the yolk using a combination of the following: 120 pg synthetic human *NPM1* mRNA, 100 pg of *NPMc+* mRNA, 50 pg of *dkk1b* mRNA and 10 ng of SBmo-*npm1a*; mRNAs and morpholino were diluted in Danieu medium (8 mM NaCl, 0.7mM KCl, 0.4 mM MgSO<sub>4</sub>, 0.6 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 5.0 mM HEPES pH 7.6) with 1% rhodamine-dextran to assess the accuracy of the injection procedure. Injected and control *gata1:dsRED* transgenic embryos were directly viewed at the microscope at indicated stages.

## **Whole-mount in situ hybridization**

The mRNA probes were synthesized as follows: 1 µg of each vector was lineared with the corresponding restriction enzyme and *in vitro* transcribed with T7, T3 or SP6

RNA polymerase as reported in table 3. To produce digoxigenin (DIG) labeled mRNA probes the DIG-RNA labeling mix (Roche) was used.

**Table 3. Vector name, restriction enzyme and RNA polymerase used for probe synthesis**

Probe	Vector	Restriction Enzyme	RNA Polymerase
<i>rx3</i>	pBS-SK	XbaI	T7
<i>pax2a</i>	pGEM-3 zf(+/-)	BamHI	T7
<i>ntl</i>	pBS-KS	HindIII	T7
<i>dlx3</i>	pBS-SK	XbaI	T7
<i>hgg1</i>	pBS-SK	XhoI	T3
<i>dkk1b</i>	pSPORT1	Sall	SP6
<i>gata2</i>	pCRII-TOPO	BamHI	SP6
<i>pu.1</i>	pBK-CMV	EcoRI	T7
<i>tal1</i>	pBK-CMV	Sall	T7
<i>lmo2</i>	pBK-CMV	EcoRI	T7
<i>l-plastin</i>	pSPORT1	EcoRI	SP6

Embryos were dechorionated at indicated stages using pronase (Sigma) diluted in E3 water and fixed overnight at 4°C with 4% paraformaldehyde (PFA) in phosphate-saline buffer (PBS) solution. Whole mount in situ hybridization was performed as previously described by Thisse et al. [123]. Probes were recognized by an alkaline phosphatase conjugated anti-Digoxigenin antibody (Roche) and signal was detected with NBT/BCIP solutions. Embryos were then immersed in 85% glycerol, flat-mounted and viewed with a Leica stereomicroscope.

## **Immunoperoxidase staining**

In Tg(*TOP:GFP*) and Tg(*mpx:GFP*) transgenic embryos, the GFP expression was revealed with an antibody against GFP (TP401, Torrey Pines Biolabs) and subsequently by colorimetric reaction using peroxidase, conjugated to the secondary antibody, and 3,3'-Diaminobenzidine (DAB).

## **Cell lines**

EML-C1, purchased from ATCC (CRL-11691), and derived cell lines were maintained in Iscove's Modified Dulbecco Medium (IMDM) medium supplemented with 20% (v/v) of heat inactivated horse serum, 15% (v/v) of BHK/MKL conditioned medium as a source of SCF, 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin. OCI-AML2 and OCI-AML3 were maintained in  $\alpha$ -MEM medium supplemented with 20% heat inactivated calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

Phoenix cells were maintained in DMEM medium supplemented with 10% heat inactivated calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin and transfected with MSCV-GFP-empty vector, MSCV-GFP-NPMc+, pBABE-puro-empty vector and pBABE-puro-DKK1 plasmids following the manufacturer's protocol. Cell supernatant containing retroviral particles was collected and concentrated by addition of PEG 8000 overnight and pelleted for 45 minutes at 3000 RPM. EML-C1 cells were infected with spin infection in 24-well plates that were centrifuged for 45 minutes at 1800 RPM. Infected cells were then sorted with an Aria Cell Sorter (BD Bioscience) or selected by administration of puromycin.

## **Dkk1 cloning**

p53<sup>-/-</sup> MEFs mRNA was retrotranscribed using the ImProm-II™ Reverse Transcription System (Promega), following manufacturer's instruction. Full-length cDNA was amplified by PCR using the Phusion high-fidelity DNA polymerase kit (NEB). PCR primers were designed to carry EcoRI and BamHI restriction enzyme recognition sites at their 5' end. Sequences of primers are the following: forDkk1 - 5'-CGGGATCCCGATGATGGTTGTGTGTGCAGC-3' and revDkk1 - 5'-CGGAATTCGGTTAGTGTCTCTGGCAGGTGT-3'. PCR products were separated in a 1% agarose gel and the band corresponded to the expected length of 840nt was cut and the DNA was isolated using the QIAquick PCR purification kit (QIAGEN) following manufacturer's instruction. Insert and pBABE DNA were digested with EcoRI and BamHI restriction enzymes for 90 minutes at 37°C and enzymes were inactivated by 20 minutes at 65°C. In order to avoid empty plasmid reformation plasmid, linearized vector was dephosphorylated by Antarctic Phosphatase (NEB). The ligation reaction was performed by incubating 100 ng of digested and dephosphorylated vector and 16 ng of digested insert with T4 DNA ligase (NEB) overnight at 16°C. Ligation product was transformed in bacterial competent cells; colonies were grown in 2 ml of LB media with 100 µg/ml of ampicillin overnight at 37°C on a shaking incubator; DNA plasmid was extracted using the QIAprep Spin Miniprep kit (QIAGEN) and digested with EcoRI and BamHI in order to control that the insert was present. Further, a DNA sequencing was done in order to control the correct sequence of the insert.

## **Myeloid cell differentiation**

EML-C1 cells were differentiated along the myeloid lineage in IMDM medium supplemented with 20% (v/v) horse serum, 8% BHK/MKL conditioned medium as SCF source, 8% WEHI 3B conditioned medium as IL3 source, and 5 µM of all-trans

retinoic acid (ATRA) (medium A). Cells treated in medium A for three days differentiate into promyelocytes. Cells were then washed and further differentiated in IMDM medium supplemented with 20% horse serum and 10ng/ml of GM-CSF (Peprotech) (medium B).

### **Flow cytometry and FACS analysis**

To assess the expression of surface markers, cells were harvested, washed twice with cold 1X PBS, blocked for 30 minutes with FcR blocking reagent (Miltenyi Biotech) diluted 1:100 in 2% BSA 1X PBS and incubated for 30 minutes with following fluorochrome-conjugated antibodies: anti-mouse c-kit-PE, anti-mouse Sca-1-PE-Cy7, anti-mouse CD11b-PE-Cy7, anti-mouse Gr-1-PE-Cy7 (eBioscience). Anti-mouse IgG2b-PE and anti-mouse IgG2a/b-PE-Cy7 were used as isotype controls. Antibodies were diluted 1:100 (PE-conjugated antibodies) or 1:200 (PE-Cy7 - conjugated antibodies) in a volume of 100  $\mu$ l. Cells were then washed twice with cold PBS and analyzed with a BD FACScalibur flow cytometer (BD Bioscience). Analyses were performed and elaborated using CellQuest software (BD Bioscience).

### **Western blot**

Cells were harvested and washed three times in PBS, lysed in laemmli buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.1%  $\beta$ -mercaptoethanol) and stored at -20°C. 15 to 30  $\mu$ g of whole cell lysate were loaded in 10-12% sodium dodecyl sulfate - polyacrylamide gels and separated through gel electrophoresis (SDS-PAGE). Separated proteins were then transferred to Protrane nitrocellulose membranes (GE Healthcare) or PVDF membranes (Millipore) for antibody probing. Membranes were incubated with either 5% non-fat milk or 5% BSA in TBST for one hour at room temperature (RT) or overnight at 4°C, then incubated for variable times with the

suitable antibodies diluted in either 5% non-fat milk or 5% BSA in 1X TBST, washed with TBST and incubated with a dilution of 1:20000 of secondary antibody for one hour at RT. The antibody was then visualized using enhanced chemiluminescence (ECL) western blotting detection reagents (Amersham) followed by autoradiography. These antibodies were used: T26 mouse anti-NPMc+ [124], mouse anti-CyclinD1 (1:2000, Cell signaling, cat. 2926), rabbit anti-phosphoPKC (1:1000, Cell signaling cat. 9371), rabbit anti-Dkk1 (1:500, Santa Cruz Biotechnology, cat. sc-25516), mouse anti-vinculin (1:10000 Sigma).

### Quantitative real-time polymerase chain reaction (qPCR)

Cells were harvested, washed twice in cold PBS and RNA was extracted using the QIAGEN RNeasy kit. RNA was quantified with Nanodrop and 500 ng – 1 µg of template RNA was retrotranscribed into cDNA using random primers and the ImProm-II™ Reverse Transcription System (Promega). 20 ng of the resulting cDNA were used for each real-time PCR reaction with 0.4 µM primers, 10 µl of SYBR Green Fast Reaction Mix (Applied Biosystem) diluted in a final volume of 20 µl. Accumulation of fluorescent products was monitored by real-time PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems). Sequences of primers are reported in tables 4, 5 and 6.

**Table 4. Sequences of primers used to analyzed Wnt target genes and Wnt components in murine cells**

	Forward	Reverse
<b>Axin1</b>	GGGCCCCCTCAAGTAGAC	CCCTCCAAGATCCATACCTG
<b>Axin2</b>	GGTTCGGGCTATGTCTTTGC	CAGTGCGTCGCTGGATAACTC
<b>Tcf7</b>	CAGCTCCCCATACTGTGAG	TGCTGTCTATATCCGCAGGAA

<b>Ctnnb</b>	GCAGCAGCAGTTTGTGGA	TGTGGAGAGCTCCAGTACACC
<b>Gsk3<math>\beta</math></b>	TTCTACAGGACAAGCGATTTAAGA	CGGACTATGTTACAGTGGTCTAGC
<b>Dvl</b>	ACTTCACCCTCCCTCGAAA	GAGGAGCCAGGGTAAGCAG
<b>Ccnd1</b>	GAGATTGTGCCATCCATGC	CTCCTCTTCGCACTTCTGCT
<b>Tbp</b>	CTGGAATTGTACCGCAGCTT	TCCTGTGCACACCATTTTTTC

**Table 5. Sequences of primers used to analyze Wnt target genes and Wnt components in patients' blasts.**

	Forward	Reverse
<b>MYC</b>	CACCAGCAGCGACTCTGA	GATCCAGACTCTGACCTTTTGC
<b>CCND1</b>	GAAGATCGTCGCCACCTG	GACCTCCTCCTCGCACTTCT
<b>AXIN2</b>	ACAACAGCATTGTCTCCAAGCAGC	GCGCCTGGTCAAACATGATGGAAT
<b>AXIN1</b>	ATGGAGCTCTCCGAGACAGA	TAGTACGCCACAACGATGCT
<b>CTNNB</b>	GCTTTCAGTTGAGCTGACCA	CAAGTCCAAGATCAGCAGTCTC
<b>TBP</b>	CGGCTGTTTAACTTCGCTTC	CACACGCCAAGAAACAGTGA

**Table 6. Sequences of primers used to analyze Hox genes in murine cells**

	Forward	Reverse
<b>HoxA1</b>	CCTTGGCAGTGGCGACTCT	GCGCAGGATTGGAAAGTTGT
<b>HoxA4</b>	CCGGAGAATGAAGTGAAGAAA	GCCGAGGCAGTGTGGAA
<b>HoxA5</b>	TAGTTCCGTGAGCGAACAATTC	GCTGAGATCCATGCCATTGTAG
<b>HoxA6</b>	CCTATTTTGTGAATCCCCTTTCC	CAGCTGGCCCAAGAAGGA
<b>HoxA7</b>	ACGCGCTTTTTAGCAAATATACG	GGGTGCAAAGGAGCAAGAAG
<b>HoxA9</b>	CCGAACACCCCGACTTCA	TTCCACGAGGCACCAAACA
<b>HoxA10</b>	CACAGGCCACTTCGTGTTCTT	TTGTCCGCAGCATCGTAGAG

<b>HoxB2</b>	GCTCGCCGAGTGTCTGACTT	AATGTCGACTCCTTGATTGATGAA
<b>HoxB5</b>	GGGCAGACTCCACAGATATTCC	GGGTCAGGTAGCGATTGAAGTG
<b>HoxB6</b>	TTCCTATTTCTGGAAGTCCACCTT	CCGCATAGCCAGACGAGTAGA
<b>HoxB9</b>	TGTCCATTTCTGGGACGCTTA	GAACACCGGCGCTTTGG

### **Murine primary stroma**

Bones of 6 to 8 weeks old C57BL/6J mice were taken and crushed. The resulting cell suspension was passed through a 70  $\mu$ M cell strainer (BD Bioscience), cells were washed and resuspended in IMDM medium supplemented with 12.5% heat inactivated calf serum, 12.5% heat inactivated horse serum, 2 mM L-glutamine,  $5 \times 10^{-5}$  M  $\beta$ -mercaptoethanol and  $5 \times 10^{-7}$  M hydrocortisone (Sigma), 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin. Cells were plated in 15 mm Petri dishes and medium was changed once a week to support growth of bone marrow stroma cells.

### **Long-term culture-initiating colonies (LTC-IC)**

Primary stromal cells were seeded in 6-well plates at a concentration of 500000 cells /ml. After two days, cells were irradiated with 15 Gy using a Faxitron 43855F and left in culture for another 2 days. After this time the medium was changed and NPMc+ or control MSCV cells were seeded on top of them; half of the medium was changed once a week during the long-term culture assay. After 6 weeks, cells were harvested and diluted 1 to 10 in M3236 methylcellulose containing medium (Stem Cell technologies) supplemented with 20% heat-inactivated horse serum, 50 ng/ml of SCF, 2 ng/ml of IL-3, 2 ng/ml of IL-6, 10 ng/ml of GM-CSF (Peprotech) and seeded in 35 mm culture plates for 7 days.



### **Colony-forming unit (CFU) assays**

Cells were cultured at the density of  $1.0 \times 10^3$ /ml in M3236 methylcellulose containing medium supplemented with 20% heat-inactivated horse serum and either 50 ng/ml of SCF for CFU-Blast or 50 ng/ml of SCF and 2 ng/ml of IL-3 for CFU-GM. Colonies were counted with ImageJ software after 7 (CFU-GM) or 14 days (CFU-Blast).

### **BrdU incorporation and cell cycle analysis**

Cells were incubated in growth medium containing 33  $\mu$ M of bromodeoxyuridine (BrdU) for 15 minutes, then washed in PBS and permeabilized in cold 70% ethanol for 30 minutes. Cells were washed, denatured with 2 N HCl (denaturing solution) for 25 minutes at RT and neutralized with 0.1M Sodium Borate for 2 minutes at RT. They were then incubated in a 1:5 dilution of mouse anti-BrdU antibody in 1% BSA for 1 hour at RT, washed, incubated in a 1:50 dilution of Cy3-conjugated anti-mouse secondary antibody in 1% BSA for 1 hour at RT, resuspended in a solution of 2.5  $\mu$ g/ml of propidium iodide (PI) and 250  $\mu$ g/ml RNase and incubated overnight at 4°C. BrdU incorporation was read with a BD FACScalibur flow cytometer (BD Bioscience). Analyses were performed and elaborated using CellQuest software (BD Bioscience).

### **Immunoprecipitation**

OCI-AML2 and OCI-AML3 cells were harvested, washed in cold PBS and lysed in the indicated buffer supplemented with protease and phosphatase inhibitors. Whole cell lysates were quantified with Bradford and aliquots of 1 mg were diluted in a total volume of 600  $\mu$ l in the corresponding buffer. 4  $\mu$ g of anti-NPM1 (Invitrogen), anti-NPMc+ [124], anti-GRP78 (Abcam, cat. ab21685) or control antibodies (anti-V5, santa

cruz, cat. sc-271944; anti-flag, Sigma, cat. F3165) were added to the solution and incubated overnight at 4°C. The following day, 50 µl of sepharose conjugated beads or dynabeads were resuspended in the corresponding buffer and added to the solution for 1 hour. Beads were pulled down, washed three times with the corresponding buffer and the immunoprecipitate was separated from the beads by adding 30 µl of blue laemmli (loading buffer – laemmli buffer supplemented with bromophenol blue) and incubated for 5 minutes at 95°C. Samples were then loaded on a polyacrilamide gel for coomassie staining using the Novex Colloidal Blue Staining kit (Invitrogen) or for western blot.

The composition of buffers used for protein work was:

- Buffer1: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1mM DTT, 0.5% NP-40
- Buffer2: 25 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.5% NP-40
- Modified RIPA: 50 mM Tris-HCl pH 7.6, 150 mM NaCl, 0.5% NP-40
- RIPA: 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate

## **RESULTS**



## **1. NPM1 and NPMc+ modulate Wnt signaling in zebrafish development and primitive hematopoiesis**

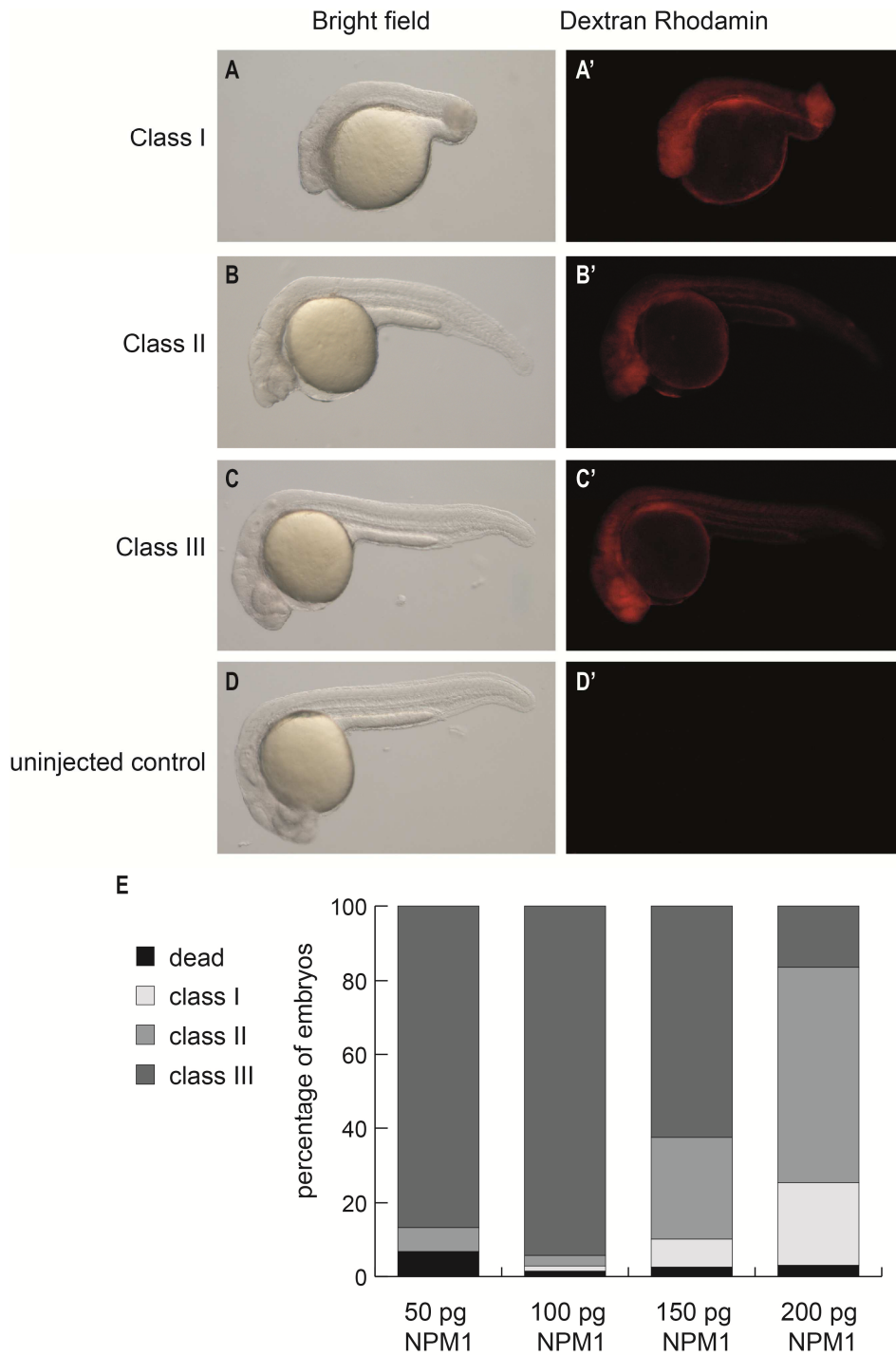
### **1.1. Effect of NPM1 and NPMc+ expression on zebrafish morphology**

Previous works in our group revealed that PRDM5, a transcription factor belonging to the PRDM gene family, negatively modulates Wnt signaling during zebrafish development [125]. Further mass spectrometry analysis showed that PRDM5 interacts with NPM1 (data not published). We therefore decided to analyze whether NPM1 and its leukemogenic mutant NPMc+ could in turn modulate Wnt signaling.

We injected increasing amount of human *NPM1* and *NPMc+* mRNA in one-cell stage embryos, in order to identify the maximum quantity of mRNA that gives a phenotype without a toxic effect. Embryos were injected with 50 pg, 100 pg, 150 pg and 200 pg of either *NPM1* and *NPMc+* mRNAs and their morphology was analyzed at 24 hours post fertilization (hpf).

As reported in figure 8, *NPM1* injected embryos were divided into 3 classes based on their morphology. The first class was composed of embryos showing the strongest phenotype, namely a small head and small tail, in some cases with unrecognizable anatomical structures (fig. 8A). These embryos might be affected by the injection itself or their morphology was the result of a large amount of mRNA that was absorbed from the yolk into the developing embryo, impairing its early development. The second class included embryos which were partially affected by mRNA injection (intermediate phenotype) and showed a general reduction of the body and of the tail in particular (fig. 8B). The third class was composed of embryos which did not display any major morphological abnormalities and were quite similar to the wild-type (fig.

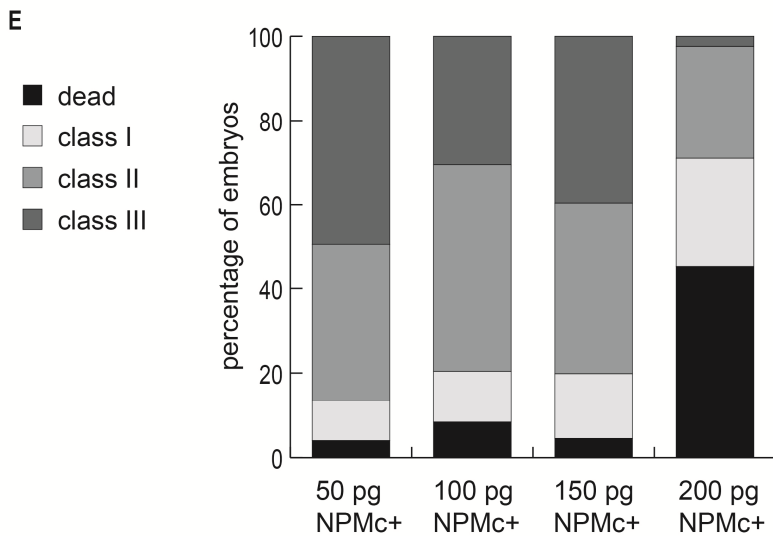
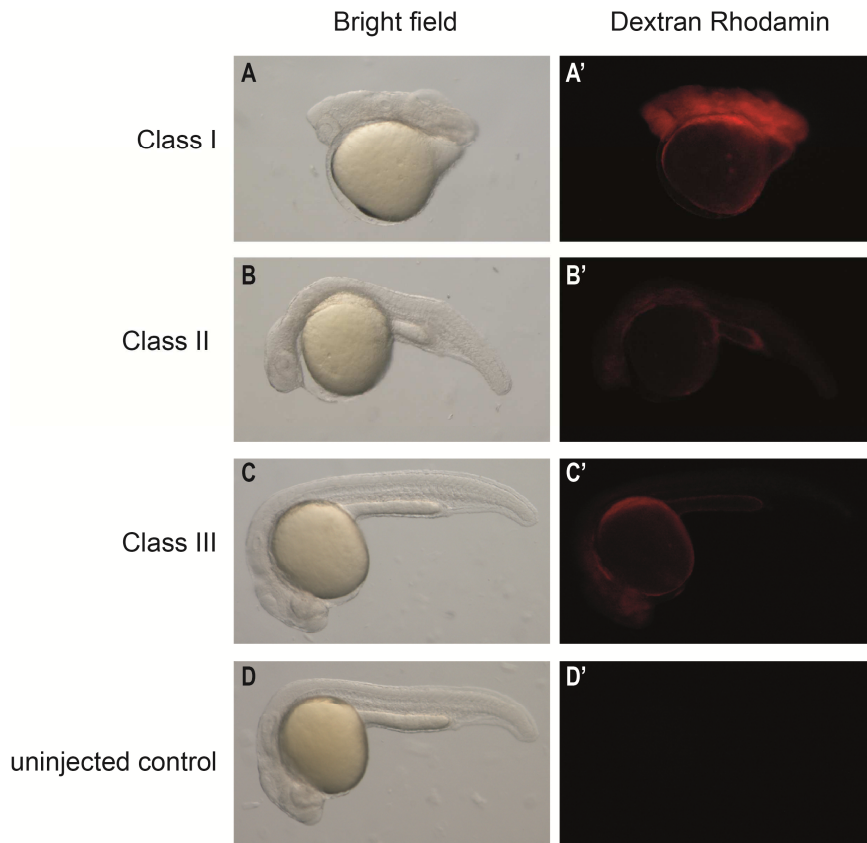
8C-D). Around 120-150 pg of *in vitro* transcribed mRNA of human *NPM1* were injected into one-cell stage zebrafish embryos in the following experiments, representing the concentration that allowed us to obtain a mild phenotype without any toxic effect.



**Figure 8. *NPM1* mRNA injection in zebrafish embryos.** Wild-type embryos were injected at one-cell stage with increasing amount of *NPM1* mRNA. Embryos were observed at 24 hpf and classified according to their morphology. Stronger phenotypes correspond to a stronger rhodamine signal. A-C:

examples of embryo's morphology for each class; D: uninjected control; E. percentage of embryos classified each morphological classes upon injection of 50 pg, 100 pg, 150 pg or 200 pg of *NPM1* mRNA.

In parallel, embryos were injected with 4 quantities of *in vitro* transcribed mRNA of the most common *NPM1* mutation isolated from AML patients (mutation A), which we referred to as *NPMc+*: *NPMc+* mRNA injected embryos were divided in three classes, depending on their morphology. Class I comprised all embryos with the strongest phenotype, frequently with unrecognizable body structures (fig. 9A). Class II was composed of embryos with defects in the body and the head: they were characterized by a shorter body, a stocky tail and an enlarged posterior hematopoietic region, located posteriorly to the end of the yolk extension (fig. 9B). Class III included embryos similar to wild-type (fig. 9C). *NPMc+* injection resulted in a stronger phenotype when injected in the same concentration as the wild-type, therefore a concentration of 100 pg was used to inject one cell-stage embryos in the following studies.



**Figure 9. *NPMc+* mRNA injection in zebrafish embryos.** Wild-type embryos were injected at one-cell stage with increasing amount of *NPMc+* mRNA. Embryos were observed at 24 hpf and classified according to their morphology. Stronger phenotypes correspond to a stronger rhodamine signal. A-C: examples of embryo's morphology for each class; D: uninjected control; E. percentage of embryos classified each morphological classes upon injection of 50 pg, 100 pg, 150 pg or 200 pg of *NPMc+* mRNA.

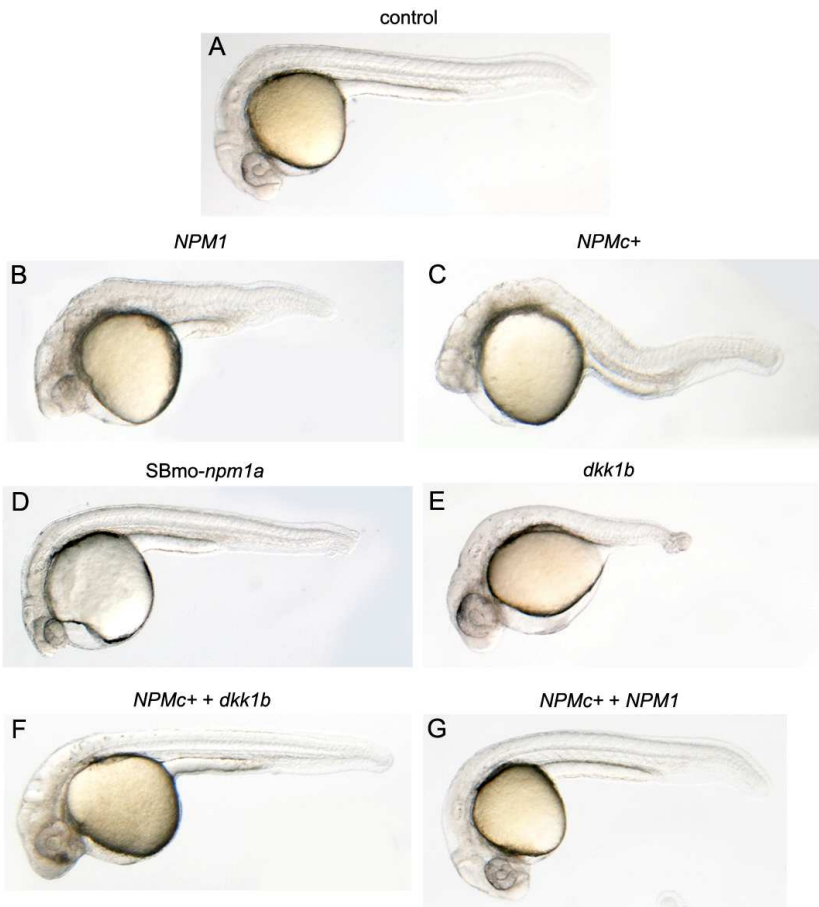
To assess the hypothesis of an involvement of NPM1 and eventually of its mutant *NPMc+* in Wnt modulation, we compared the effect of NPM1 and *NPMc+* expression and endogenous *npm1a* knockdown to *dkk1b* mRNA injection, whose effect in zebrafish development is well characterized [126]. Dkk1 is a known inhibitor of the



canonical Wnt pathway [67], whose overexpression in zebrafish leads to the anteriorization of the embryos body resulting in smaller tails and bigger heads.

At 28 hpf, *NPM1* injected embryos showed a reduction of the body size, as compared to control embryos at the same developmental stage (fig. 10B). After the injection of 100pg of *NPMc+* mRNA, we indeed observed embryos characterized by a smaller head compared to wild-type controls, in some cases lacking eyes, and a globally posteriorized phenotype (Fig. 10C). Silencing of the endogenous homologue of NPM1, *npm1a*, was achieved through the injection of 10 ng of a splice-blocking morpholino oligonucleotide (SBmo-*npm1a*) designed to recognize the boundary between *npm1a* exon 2 and intron 2: morphants (morpholino-injected embryos) did not display a strong phenotype but only a minor reduction of head and eyes size (fig. 10D). As expected, overexpression of *dkk1b* resulted in anteriorized embryos, which displayed defects in the posterior region of their body (fig. 10E).

Interestingly, co-injection of *NPMc+* and *dkk1b* partially rescued the anteriorized phenotype giving rise to embryos with a big head compared to controls but with milder defects in the tail and the posterior region of the embryos (fig. 10F). These observations suggested a positive modulation of Wnt signaling by *NPMc+* which could partially counteract the effect of the Wnt inhibitor *dkk1b*.



**Figure 10. Morphology of injected embryos at 28 hpf.** Zebrafish embryos were uninjected (A), injected with 120 pg *NPM1* mRNA (B), 100pg *NPMc+* mRNA (C), 10 ng *npm1a*-SBmo (D), 50 pg *dkk1b* mRNA (E), a mix of *NPMc+* and *dkk1b* mRNAs (F) and a mix of *NPMc+* and *NPM1* mRNAs (G).

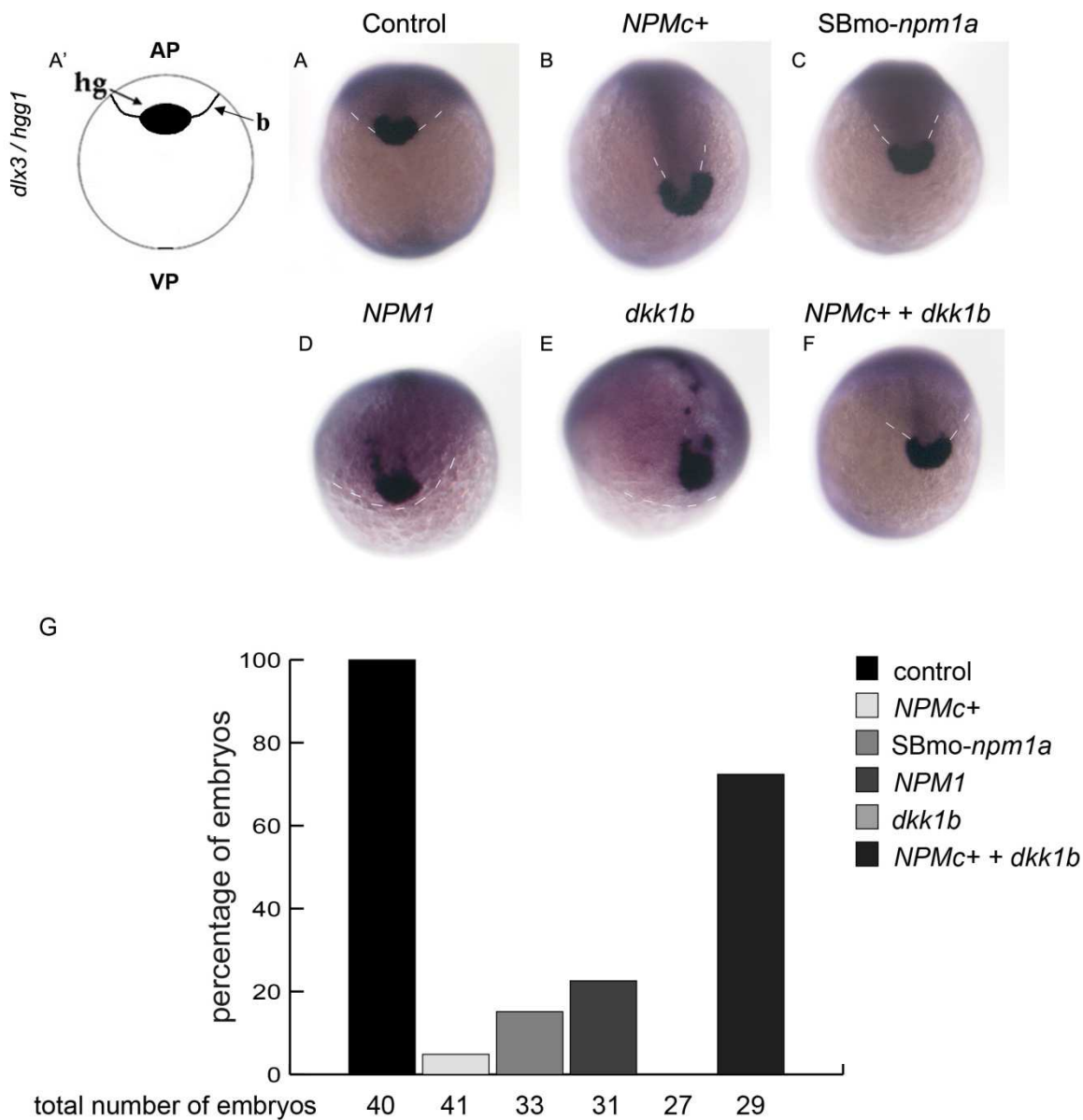
*NPMc+* may well exert a dominant negative effect on the wild-type function [27]. Therefore, we investigated whether co-injection of *NPM1* with *NPMc+* could rescue the *NPMc+* phenotype. Embryos expressing both *NPMc+* and *NPM1* showed a wild-type morphology (fig. 10G), confirming the opposite effect of the two mRNAs on zebrafish development and supporting the hypothesis of a dominant negative function of *NPMc+* on the wild-type counterpart.

## 1.2. NPM1 and NPMc+ modulate CE movements during zebrafish gastrulation

The phenotype observed after NPM1 or NPMc+ expression could be the consequence of a block of convergence and extension (CE) movements during gastrulation, which are partially controlled by non-canonical Wnt pathway, in particular the planar cell polarity (PCP) pathway [76]. To test this hypothesis, whole mount *in situ* experiments with a cocktail of two cRNA probes, *hgg1* and *dlx3*, were performed to analyze the non-canonical Wnt pathway in NPM1 or NPMc+ expressing embryos.

In control embryos at 90% epiboly stage, *hgg1* identifies the hatching gland, which corresponds to the rostral mesendoderm. In not injected embryos, its expression was confined to the anterior part of the embryo and was centered in the border between the neural and the non neural ectoderm, highlighted by *dlx3* expression at the animal pole of the embryo (fig. 11A). Embryos injected with either *NPMc+* mRNA (fig. 11B) or SBmo-*npm1a* (fig. 11C) showed a narrower region of the future neural plate. Moreover, the hatching gland was positioned anteriorly to the neural plate border (fig. 11B-C). Conversely, the injection of *NPM1* mRNA led to an expansion of the neural plate region and the inclusion of the hatching gland in the presumptive neural ectoderm (fig. 11D). Surprisingly, a similar effect was found after over-expression of *dkk1b*: the hatching gland was embodied in the neural ectoderm that completely surrounded the yolk (fig. 11E); although *dkk1b* is an inhibitor of the canonical pathway, it is also involved in the PCP pathway [126]. Overexpression of *dkk1b* led to an expansion of the future neural ectoderm which enclosed the hatching gland. *Dkk1b* overexpression was able to partially revert the effect of NPMc+: embryos still presented an anteriorization of the hatching gland with respect to non-injected

embryos but the neural plate region shrank to normal size (fig. 11F). Percentage of embryos that maintained wild-type phenotype is reported in figure 11G.

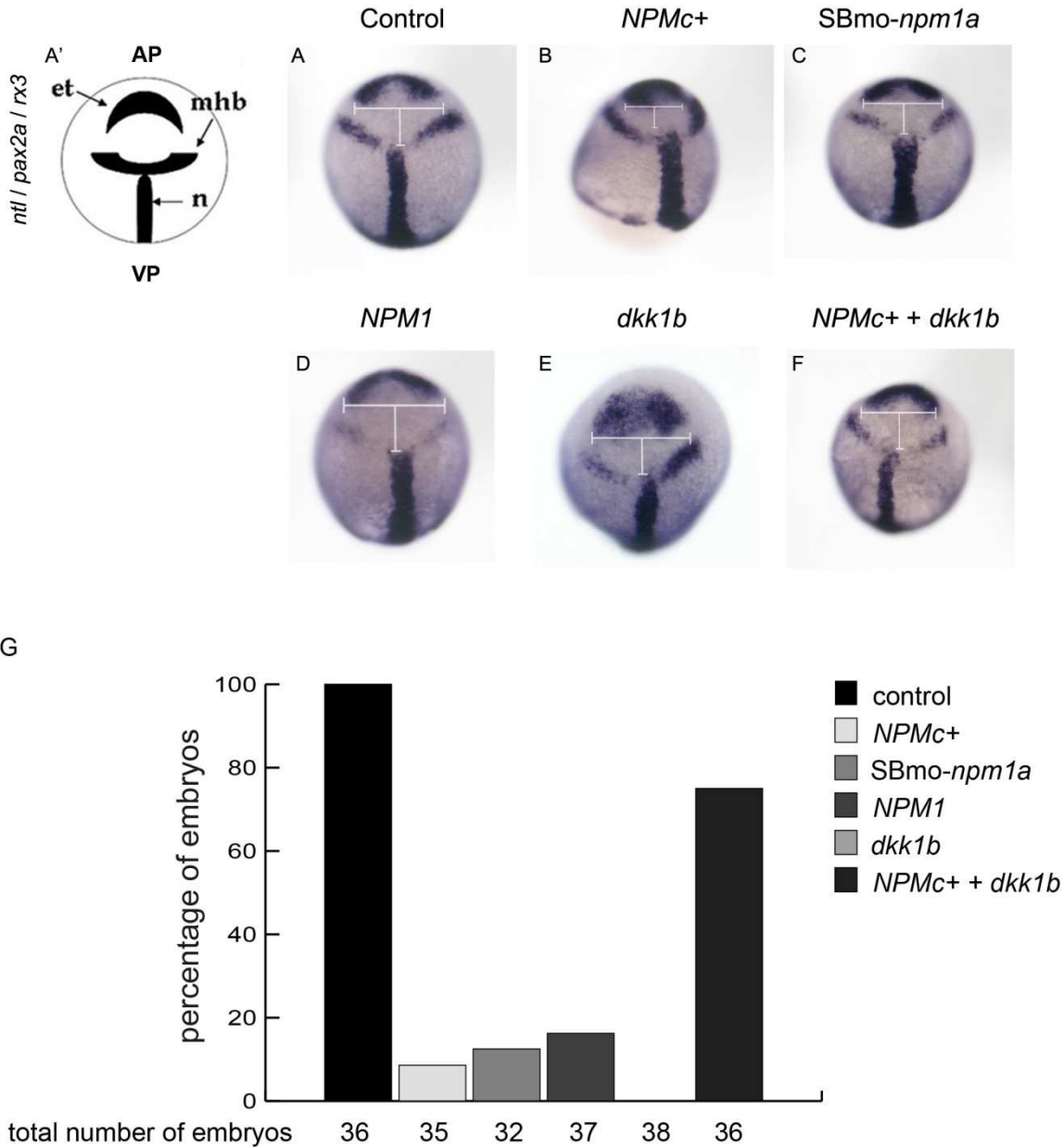


**Figure 11. Analysis of convergence and extension movements.** A-F: whole-mount *in situ* hybridization for *hgg1* and *dlx3*, marking the rostral mesendoderm (hg) and the neural border (b) respectively (A). White dashed lines mark the *dlx3* signal, corresponding to the neural and non-neural ectoderm border. All embryos are at 90% epiboly, dorsal view. AP: animal pole; VP: ventral pole. Embryos were injected with 120 pg *NPMc+* mRNA (B), 50 pg *npm1a*-*SBmo* (C), 100 pg *NPM1* mRNA (D) 50 pg *dkk1b* mRNA (E) and co-injected with a mixture of *NPMc+* and *dkk1b* mRNAs (F). G: Percentage of embryos showing a phenotype similar to control.

### 1.3. NPMc+ modulates canonical Wnt signaling at gastrulation

Canonical Wnt signaling is involved in the formation of the anteroposterior axis of specific organs (e.g. central nervous system, retina) and of the whole embryo body [73, 127, 128]. Thus, modulation of Wnt signaling by NPM1 and NPMc+ could be revealed by a modification of the respective positions of three different regions in the anterior neuroectoderm during gastrulation of the zebrafish embryo: the eye/telencephalon, the mid-hindbrain border and the notochord, identified by the expression of *rx3*, *pax2a* and *ntl*, respectively [128].

The expression of NPMc+ and, to a lesser extent, the injection of SBmo-*npm1a* reduced the distance between the regions highlighted by the three probes, indicating a reduction of the distance between the eye/telencephalon and the mid-hindbrain border. This effect implied activation of the canonical Wnt pathway and, indeed, the effect of NPMc+ was rescued by the co-injection of *dkk1b* mRNA. The over-expression of NPM1 yielded a slight increase in the distance between the two region that do not appear to be significant when compared to control (fig. 12A-F). Percentage of embryos similar to wild-type is reported in figure 12G.

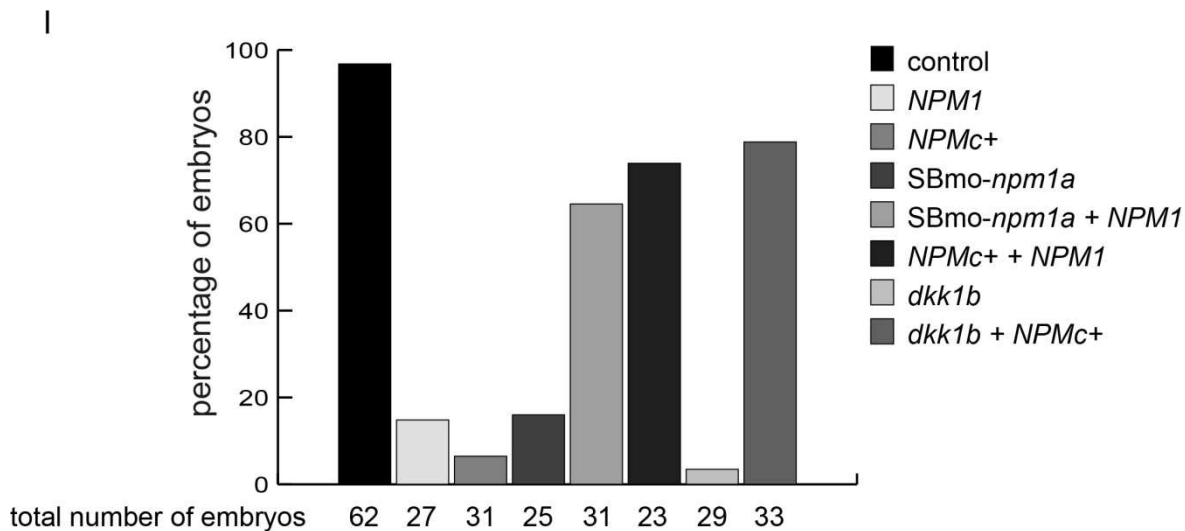
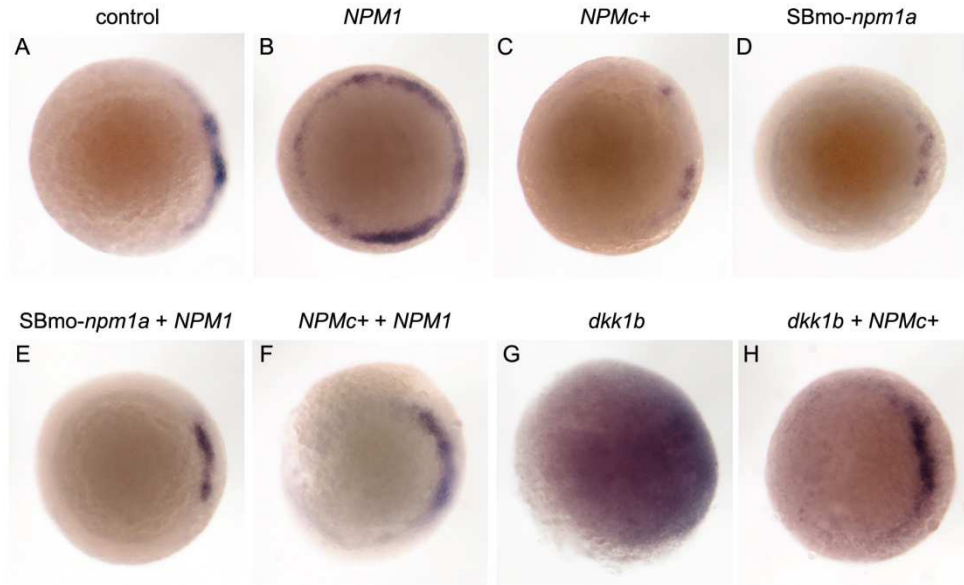


**Figure 12. Analysis of gastrulation movements.** A-F. Whole mount *in situ* hybridization for *rx3*, *pax2a* and *ntl*, markers of the eye field and telencephalon region (et), midhindbrain boundary (mhb) and notochord (n), respectively (A). White lines mark the distance between eye field and midhindbrain boundary. All embryos are at 90% epiboly, dorsal view. AP: animal pole; VP:ventral pole. Embryos were injected with 100 pg *NPMc+* mRNA (B), 10 ng *npm1a*-SBmo (C), 120 pg *NPM1* mRNA (D), 50 pg *dkk1b* mRNA (E) and co-injected with a mixture of *NPMc+* and *dkk1b* mRNAs (F). G: Percentage of embryos showing a phenotype similar to control.

#### 1.4. NPM1 and NPMc+ regulate *dkk1b* expression at early stages of zebrafish development

Since injection of *NPM1* mRNA in zebrafish embryos resulted in a phenotype similar to that observed after *dkk1b* overexpression, we investigated whether NPM1 and NPMc+ could have an effect on *dkk1b* expression during early stages of development. Dkk1b is indeed an inhibitor of the pathway but, at early stages of development, it is also a target gene of the pathway [73]. Therefore, we analyzed the effect of NPM1 and NPMc+ on endogenous *dkk1b* expression at 30% epiboly by *in situ* hybridization with a RNA DIG-labeled probe.

In uninjected embryos used as control, *dkk1b* was expressed dorsally, in the region that corresponds to the organizer (fig. 13A). Overexpression of *NPM1* led to a broader distribution of *dkk1b* as compared to wild-type controls: *dkk1b* expression was extended to the ventral region of the embryos (fig. 13B). On the contrary, both the expression of *NPMc+* and SBmo-*npm1a* injection strongly decreased *dkk1b* signal, which appeared milder and narrower than in control embryos (fig. 13C-D). The similarity in the effect elicited by NPMc+ expression or knockdown of the endogenous protein further suggests a dominant negative effect of the mutant over the wild-type NPM1 protein. Injection of *NPM1* mRNA rescued the effect of both *NPMc+* and SBmo-*npm1a* (fig. 13E-F), confirming that the downregulation of *dkk1b* expression was a consequence of NPMc+ expression or *npm1a* ablation. Moreover, NPMc+ could counteract the expression of exogenous *dkk1b* (fig. 13G-H), suggesting that NPMc+ could modulate the level of both endogenous and exogenous *dkk1b*. Percentage of embryos similar to wild-type is reported in figure 13I.



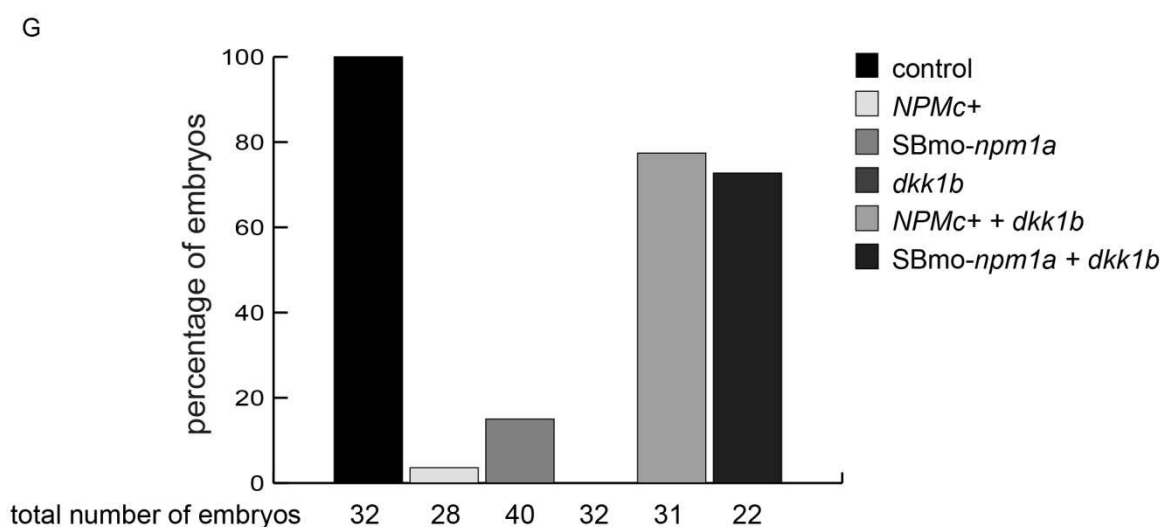
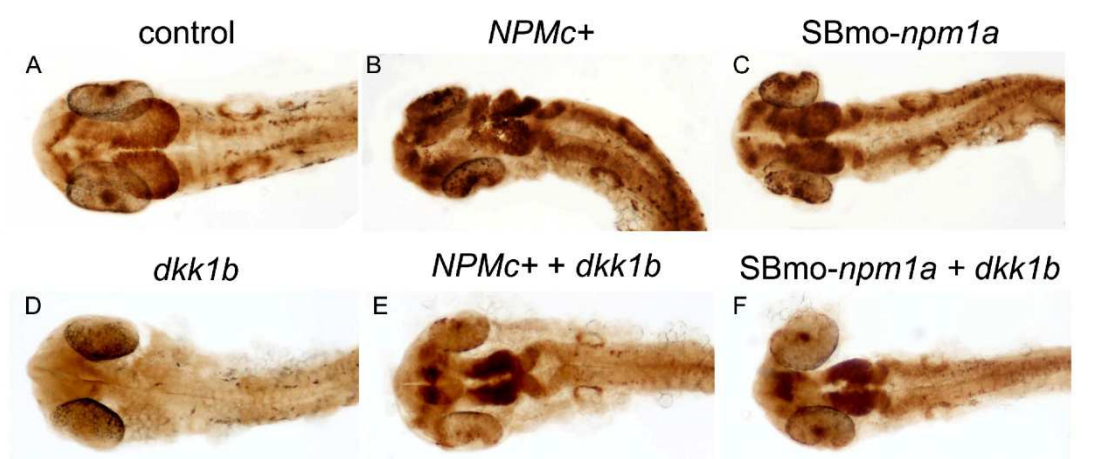
**Figure 13. *dkk1b* expression at 30% epiboly (4.7 hpf).** Whole-mount RNA *in situ* hybridization for *dkk1b* at 30% epiboly. Embryos are shown in lateral view and were injected with 120 pg *NPM1* mRNA (B), 100 pg *NPMc+* mRNA (C), 10 ng *npm1a*-SBmo (D), 50 pg *dkk1b* mRNA (G) and a combination of SBmo-*npm1a* and *NPM1* mRNA (E), *NPMc+* mRNA and *NPM1* mRNA, (F) *NPMc+* mRNA + *dkk1b* mRNA (H). I: Percentage of embryos showing a phenotype similar to control.

### 1.5. NPM1 and NPMc+ modulate canonical Wnt signaling

NPM1 and NPMc+ modulate canonical Wnt signaling at early stages of development by regulating the expression of the Wnt inhibitor *dkk1b*. To investigate whether



modulation of the pathway was maintained in older embryos, we analyzed the effect of *NPM1* and *NPMc+* injection in the Tg(*TOP:GFP*) transgenic strain. In Tg(*TOP:GFP*) fish, expression of the GFP reporter gene is controlled by four enhancers and the basal promoter of *lef1*, a  $\beta$ -catenin-dependent transcription factor [121]: the GFP signal is therefore a sign of canonical Wnt signaling activation. Due to the low level of fluorescence in the transgenic embryos, GFP expression was revealed at 28 hpf using an anti-GFP antibody and DAB staining in embryos injected with *NPMc+* mRNA, SBmo-*npm1a* or *dkk1b* mRNA. The presence of *NPMc+* increased the  $\beta$ -catenin signal (fig 14A-B), and to a lesser extent, a similar effect was obtained by the knockdown of endogenous *npm1a* with SBmo-*npm1a* (fig. 14C).



**Figure 14. Canonical Wnt activation in 28 hpf embryos.** DAB-staining for GFP expression in TOP:GFP embryos at 28 hpf. Embryos are shown in dorsal view, anterior at the left, and were injected

with 100 pg *NPMc+* mRNA (B), 10 ng *npm1a*-SBmo (C), 50 pg *dkk1b* mRNA (D) and a combination of *NPMc+* mRNA + *dkk1b* mRNA (E) and SBmo-*npm1a* + *dkk1b* mRNA (F). G: Percentage of embryos showing a phenotype similar to control.

Overexpression of *dkk1b* strongly decreased the GFP signal (fig. 14D); however, when *dkk1b* mRNA was co-injected with *NPMc+* mRNA the signal was rescued (fig. 14E), suggesting that the presence of *NPMc+* could lead to activation of Wnt signaling in more differentiated embryos as well, overcoming *dkk1b*-dependent inhibition. Moreover, downregulation of endogenous *npm1a* also counteracted the effect of *dkk1b* overexpression, as shown by the partial rescue of the GFP signal (fig. 14F). Percentage of embryos similar to wild-type is reported in figure 14G.

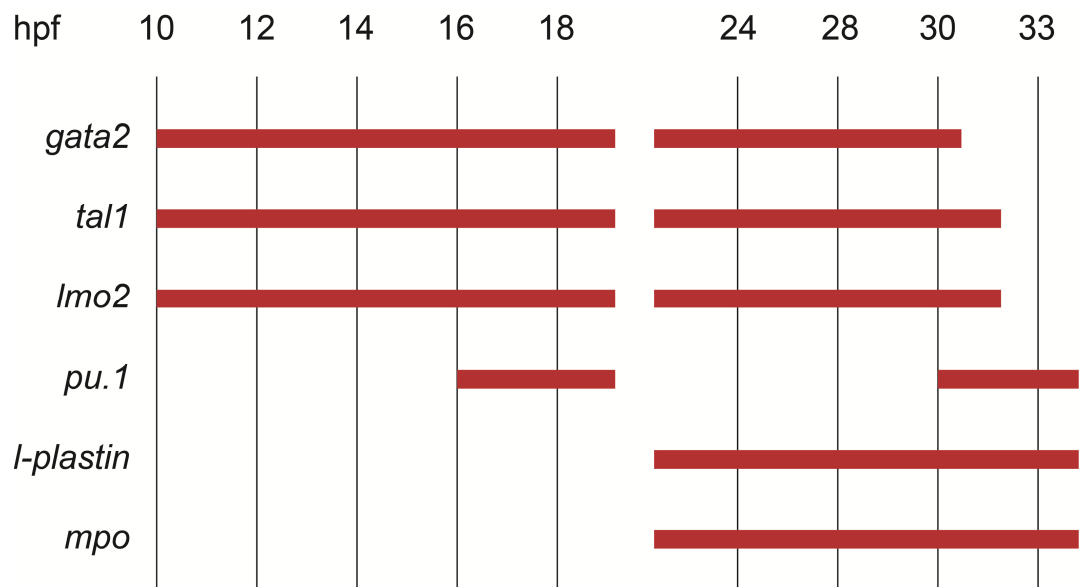
These results show that modulation of canonical Wnt signaling by NPM1 and *NPMc+* is maintained at later stages of zebrafish development.

### **1.6. Expansion of the hematopoietic progenitor pool after *NPMc+* expression is Wnt-dependent**

Since *NPMc+* is specific to AML, we next focused our attention on the hematopoietic compartment during development.

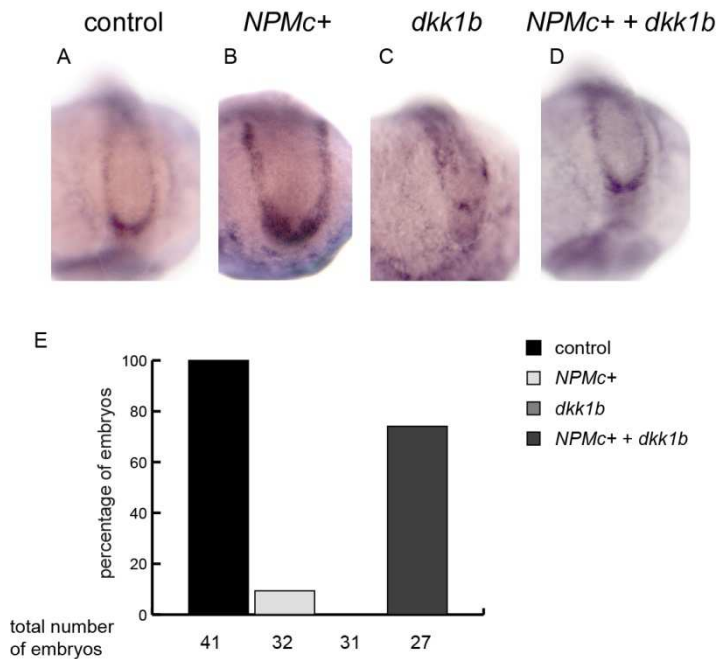
Whole mount *in situ* hybridization experiments were performed with the aim of analyzing the expression of known hematopoietic markers in zebrafish embryos expressing *NPMc+*. Hematopoietic markers were analyzed at different time points, according to their expression pattern: *gata2*, *tal1* and *lmo2* in early hematopoietic progenitors, *pu.1* in myeloid progenitors, *gata1* in cells of the erythroid lineage, *l-plastin* and *mpx* in differentiated myeloid cells, monocyte/macrophages and granulocytes, respectively [113]. A scheme of the markers used to identify different

cell types is outlined in figure 15, with the corresponding timing of expression during zebrafish development.



**Figure 15. Cartoon of hematopoietic marker expression during primitive hematopoiesis in zebrafish.** Timing of expression of hematopoietic markers is depicted for hematopoietic markers expressed in the posterior hematopoietic region of zebrafish embryos during primitive hematopoiesis.

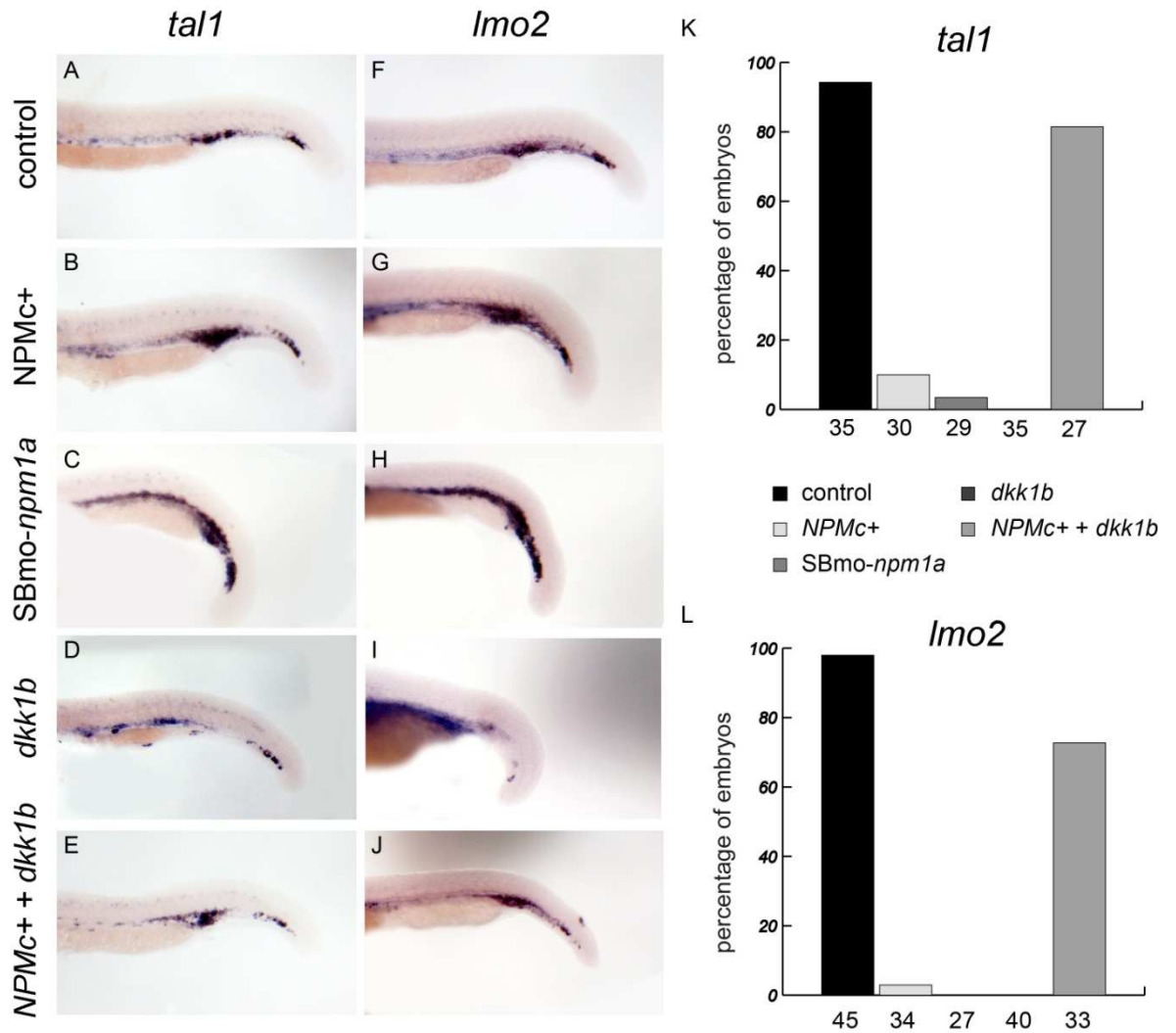
The injection of *NPMc+* in one-cell stage embryos led to increased expression of *gata2* at the stage of 15 somites (approximately 16.5 hpf), revealing an expansion of the pool of primitive progenitors (fig. 16B). The signal was, instead, faint in *dkk1b* overexpressing embryos (fig. 16C), but co-injection of *dkk1b* and *NPMc+* mRNAs rescued the phenotype (fig. 16D). Percentage of embryos showing an expression of *gata2* similar to wild-type is reported in figure 16E.



**Figure 16. Analysis of hematopoietic progenitors in 15 somite embryos.** A-D. Whole-mount *in situ* hybridization for *gata2* in 15s embryos, dorsal and posterior view. Embryos were injected with 100 pg *NPMc+* mRNA (B), 50 pg *dkk1b* mRNA (C) and co-injected with *NPMc+* and *dkk1b* mRNAs (D). E: Percentage of embryos showing a phenotype similar to control.

The other two markers that identify early progenitors, *tal1* (fig. 17A-E) and *lmo2* (fig. 17F-J), displayed the same expression pattern at 24 hpf as described above for *gata2* at 15 somites. Taken together, these data suggest that this effect of *NPMc+* depends on its capacity to activate Wnt signaling.

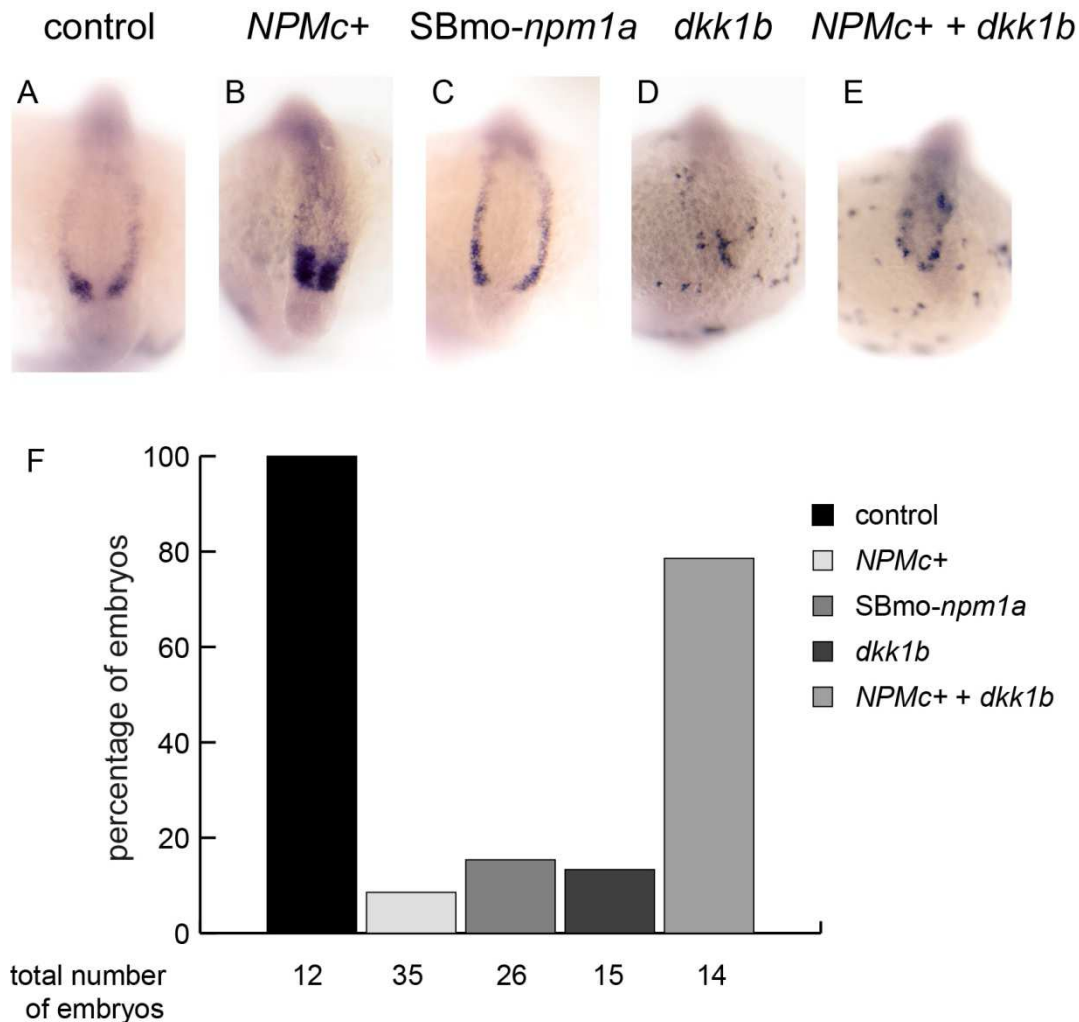
The knock down of endogenous *npm1a* had a similar effect to *NPMc+* mRNA injection on *tal1* and *lmo2* expression: both hematopoietic progenitor markers showed an increased expression (figures 17C and 17H) further supporting the hypothesis of a dominant negative effect of *NPMc+* on the wild-type. For each marker the percentage of embryos displaying unaltered phenotype is reported in figure 17K (*tal1*) and 17L (*lmo2*).



**Figure 17. Analysis of hematopoietic progenitors in 24 hpf embryos.** A-E: Whole-mount *in situ* hybridization for *tal1* in 24 hpf embryos. F-J: Whole-mount *in situ* hybridization for *lmo2* in 24 hpf embryos. Embryos are shown in lateral view, anterior to the left. Embryos were injected with 120 pg *NPMc+* mRNA (B, G), 10 ng *SBmo-npm1a* (C, H), 50 pg *dkk1b* mRNA (D, I) and co-injected with *NPMc+* and *dkk1b* mRNAs (E, J). K-L: Percentage of embryos showing a phenotype similar to control for *tal1* (K) and *lmo2* (L).

Primitive myeloid precursors, characterized by expression of the *pu.1* transcription factor [113], were studied in embryos at the stage of 15 somites. The pool of *pu.1* positive cells was strongly increased in *NPMc+* injected embryos (fig. 18B) but not affected by the injection of the morpholino against the endogenous *npm1a* (fig. 18C). *Pu.1* expression was decreased in *dkk1b* overexpressing embryos; moreover, injection of *dkk1b* mRNA led to the dispersion of the few *pu.1* positive cells in the posterior region of the embryos (fig. 18D). The co-injection of *NPMc+* with *dkk1b* only partially rescued the phenotype (fig. 18E). It appears, therefore, that *NPMc+* expression leads

to an increase in the pool of myeloid precursors, as defined by *pu.1* expression, at early stages of development. In figure 18F the percentage of embryos maintaining a phenotype similar to control is reported.

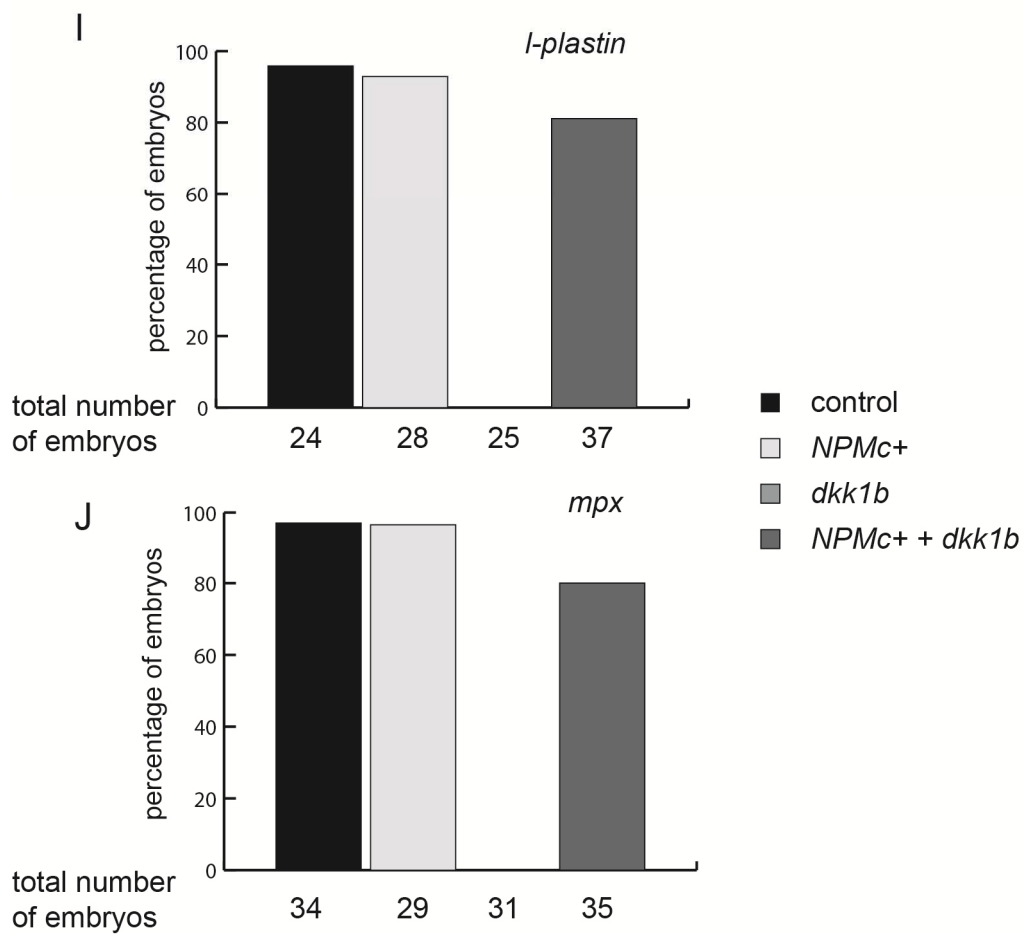
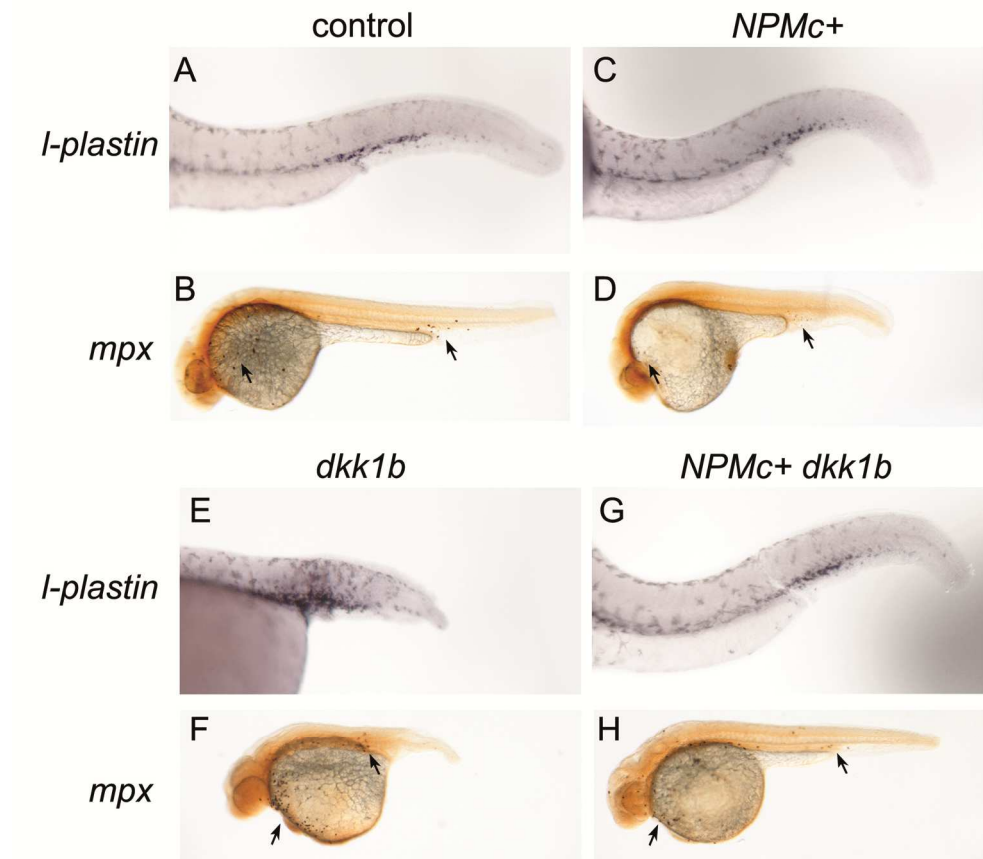


**Figure 18. Analysis of myeloid progenitors in 15 somites embryos.** A-E: Whole-mount *in situ* hybridization for *pu.1* in 15 somite embryos, dorsal and posterior view. Embryos were injected with 100 pg *NPMc+* mRNA (B), 10 ng of SBmo-*npm1a* (C), 50 pg *dkk1b* mRNA (D) and co-injected with *NPMc+* and *dkk1b* mRNAs (E). F: Percentage of embryos showing a phenotype similar to control.

In conclusion, these experiments showed that *NPMc+* expression led to an expansion of the pool of progenitor cells during primitive zebrafish haematopoiesis and this effect was rescued by overexpression of *dkk1b*, suggesting that the phenotype depends on Wnt signaling activation.

### 1.7. NPMc+ expression does not affect myeloid differentiated cells

To assess if the increase in the number of progenitor cells observed at the stage of 15 somites and 24 hpf corresponds to the expansion of one or more types of mature blood cells, we performed *in situ* hybridization experiments for markers of differentiated cells. In *NPMc+* injected embryos, the numbers of both monocytes/macrophages (*l-plastin+*) and neutrophils (*mpx+*) were similar or slightly decreased compared to wild type controls (fig. 19A-D). The injection of *dkk1b* mRNA provoked an apparent expansion of the pool of differentiated myeloid cells (fig. 19E-F), which may, however, result from the dramatic decrease in tail length. Co-injection with the mutant reverted the phenotype back to wild-type (fig. 19G-H). Percentage of embryos with control-like phenotype is reported in figure 19I for *l-plastin* and 19J for *mpx*. The expansion of the pool of hematopoietic progenitors was, therefore, not strictly connected to an increase in the number of more differentiated cells, even if *NPMc+* could rescue the effect of *dkk1b* overexpression in monocytes and neutrophils. The effect of the morpholino is still to be addressed.



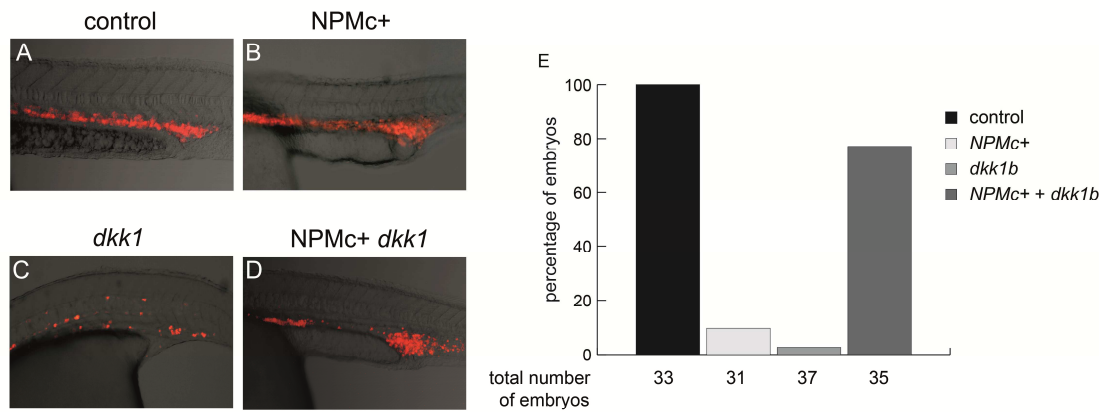
**Figure 19. Analysis of mature myeloid cells in 33 hpf embryos.** A-H. Whole-mount *in situ* hybridization for *l-plastin* (A, C, E, G) and DAB-staining for GFP in 24 hpf *mpx*:GFP embryos (B, D, F, H).



Arrows indicate areas of GFP expressing cells in *mpx:GFP* injected and control embryos. Embryos were shown in lateral view, anterior to the left. Embryos were injected with 100 pg *NPMc+* mRNA (C-D), 50 pg *dkk1* mRNA (E-F) and co-injected with *NPMc+* and *dkk1* mRNAs (G-H). I-J: Percentage of embryos showing a phenotype similar to control for *l-plastin* (I) and *mpx* (J).

### **1.8. NPMc+ partially rescues *dkk1b*-derived depletion in the erythroid lineage during primitive erythropoiesis**

The effect of *NPMc+* on erythropoiesis was investigated by expression of the corresponding mRNA in *gata1:dsRED* embryos: in this system, the expression of dsRED is under the control of the *gata1* promoter, thus marking cells that express the erythropoietic marker *gata1*. Upon *NPMc+* expression, the pool of cells expressing *gata1* was slightly increased at 24 hpf (fig. 20A, B) suggesting that the erythroid lineage was partially affected by the presence of *NPMc+*. On the contrary, the injection of *dkk1b* mRNA had a strong negative effect, with a considerable decrease of the *gata1*<sup>+</sup> cells (fig. 20C). The co-injection of the two mRNAs enhanced the expression of *gata1* compared to *dkk1b* alone, but positive cells were more concentrated in the primitive hematopoietic region (fig. 20D), confirming that *NPMc+* partially rescued the effect of overexpression of *dkk1b* in zebrafish embryos. Figure 20E shows the percentage of embryos with unaltered phenotype compared to control.



**Figure 20. Expression of the erythrocytic marker *gata1* in 24 hpf embryos.** A-D. *gata1:dsRED* embryos at 24 hpf, lateral view, anterior to the left. Embryos were injected with 100 pg *NPMc+* mRNA (B), 50 pg *dkk1b* mRNA (C) or co-injected with *NPMc+* and *dkk1b* mRNAs (D). E: Percentage of embryos showing a phenotype similar to control.

Taken together, our results suggest that both NPM1 and its leukemogenic mutant NPMc+ modulate Wnt signaling during zebrafish development, albeit in opposite ways: NPM1 attenuates Wnt-derived signals, whereas NPMc+ activates both canonical and non-canonical Wnt pathways. Furthermore, NPMc+ causes an expansion of the hematopoietic progenitor pool during primitive zebrafish hematopoiesis, and this phenotype derives from the modulation of Wnt signaling, since it is rescued by the overexpression of the Wnt inhibitor *dkk1b*.

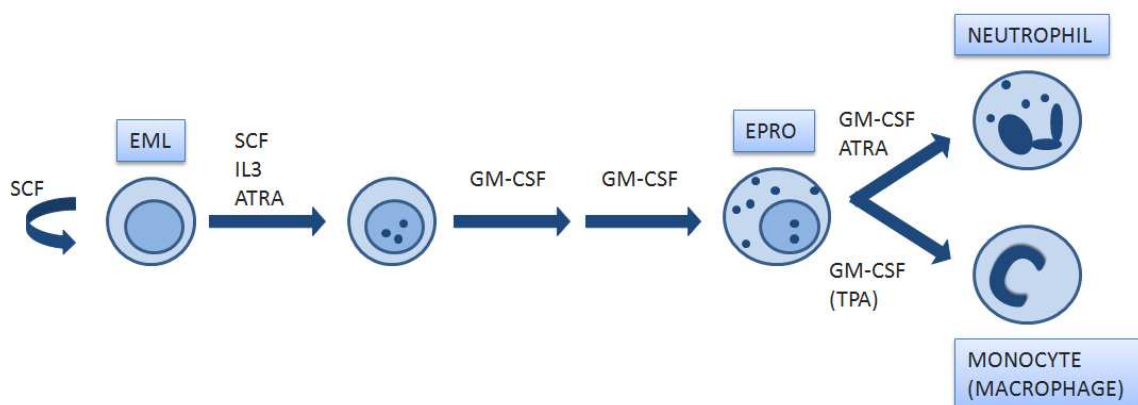
## 2. An in vitro system to stably express NPMc+

### 2.1. Characterization of NPMc+ expressing cell line

#### 2.1.1. Proliferation ability of NPMc+ expressing cells

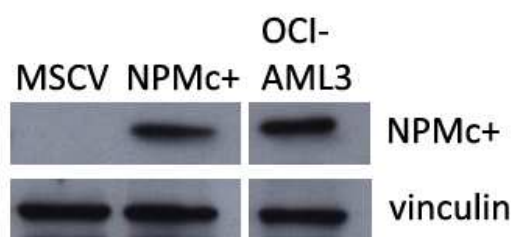
In order to better analyze the effect of the mutant on Wnt signaling in hematopoiesis, we decided to generate an *in vitro* mammalian model. To this end, we

expressed the NPM1 mutant in a murine hematopoietic stem/progenitor cell line, EML-C1, to analyze the effect of NPMc+ on proliferation, differentiation and expression of Wnt genes. EML-C1 is a Stem Cell Factor (SCF) - dependent cell line generated after expression of a dominant negative form of the retinoic acid receptor  $\alpha$  (RAR $\alpha$ ), which blocks the differentiation towards the myeloid and lymphoid lineages ,in mouse bone marrow cells [129]. Treatment with different cocktails of cytokines allows EML-C1 cells to overcome the block and differentiate into erythrocytes, monocytes/macrophages, granulocytes or lymphocytes [129, 130]. In particular, a three day treatment with retinoic acid (atRA) in presence of IL-3 and SCF (medium A) induces myeloid differentiation of EML-C1 cells to the promyelocytic stage. Differentiated cells can then be obtained in a medium containing Granulocyte Monocyte - Colony Stimulating Factor, GM-CSF, (medium B) for 3-10 days and are called EPRO (EML-derive PROmyelocytes). EPRO cells can further differentiate to neutrophils by the addition of atRA to medium B or to monocytes/macrophages with 12-O-tetradecanoylphorbol-13-acetate (TPA) administration (figure 15) [129, 130].



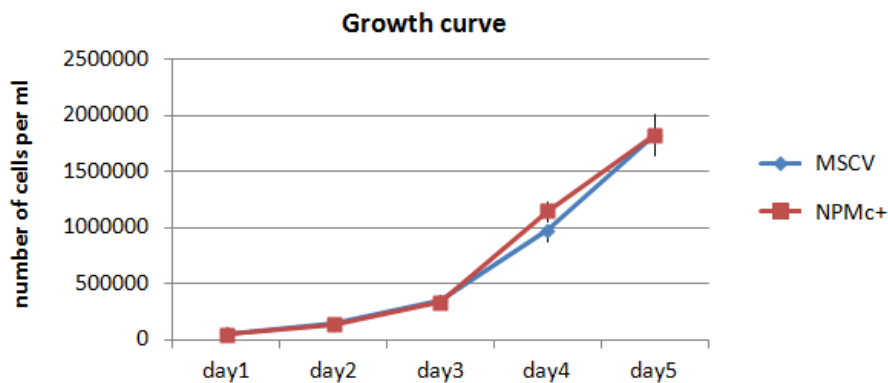
**Figure 21. Schema of EML-C1 myeloid differentiation protocol.** EML-C1 cells are maintained undifferentiated in a SCF-rich medium. EML-C1 cells may be differentiated in a IL-3, retinoic acid (atRA) rich medium (medium A). After 3 days in medium A, EML derived promyelocytes (EPRO) are maintained in a GM-CSF containing medium (medium B) and can be further differentiate to neutrophils, by the addition of ATRA, or monocytes, by the addition of 12-O-tetradecanoylphorbol-13-acetate TPA [129, 130].

EML-C1 cells were retrovirally infected with MSCV-IRES-GFP vector containing the cDNA of mutation A of NPM1 (NPMc+ cells) where the oncogene and GFP expression are driven by a 5' LTR promoter. As a control, a cell line bearing the empty vector (MSCV cells) was generated. Infected cells were isolated by FACS-sorting based on GFP expression and expression of NPMc+ was detected with western blot. As reported in figure 22, NPMc+ expression in EML cells was comparable to that observed in OCI-AML3, a patient-derived cell line which carries the NPM1 mutation A.

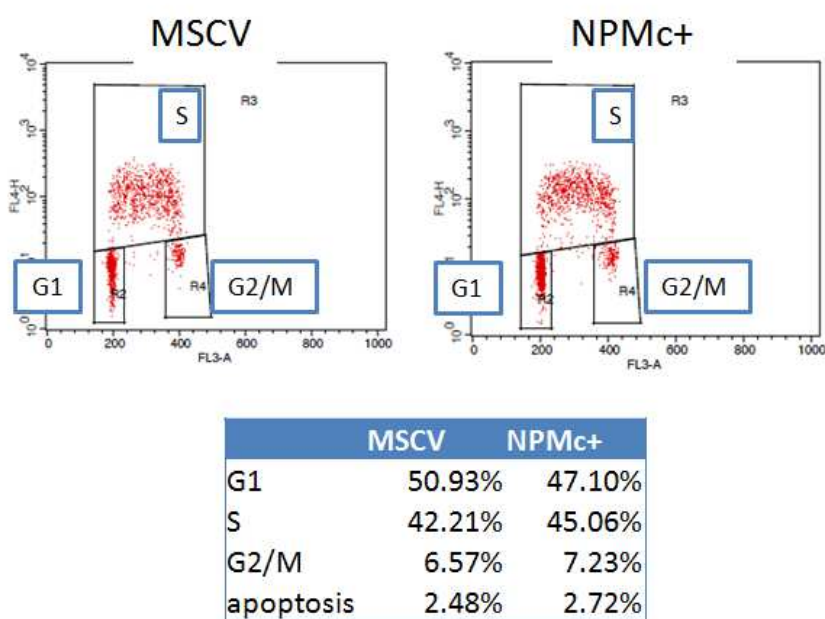


**Figure 22. Expression of NPMc+ in the MSCV-NPMc+ infected EML-C1 cells.** Western blot showed the expression levels of NPMc+ in FACS sorted pool of infected cells. MSCV cells were used as negative control and OCI-AML3 as positive control. Vinculin was used as loading control.

No differences in terms of proliferation and myeloid differentiation were observed in EML-C1 cells expressing NPMc+ as compared to the control cells. As shown in figure 23, the growth curve of NPMc+ expressing cells was comparable to control. Moreover, NPMc+ did not affect cell cycle: BrdU incorporation followed by FACS analysis did not detect any differences in cell cycle progression or apoptosis (Fig. 24).



**Figure 23. Growth curve of NPMc+ expressing cells compared to control.** NPMc+ and MSCV cells were seeded at 50000 cells/ml and counted each day. The number of NPMc+ (red line) and MSCV (blue line) cells per ml per day is reported .

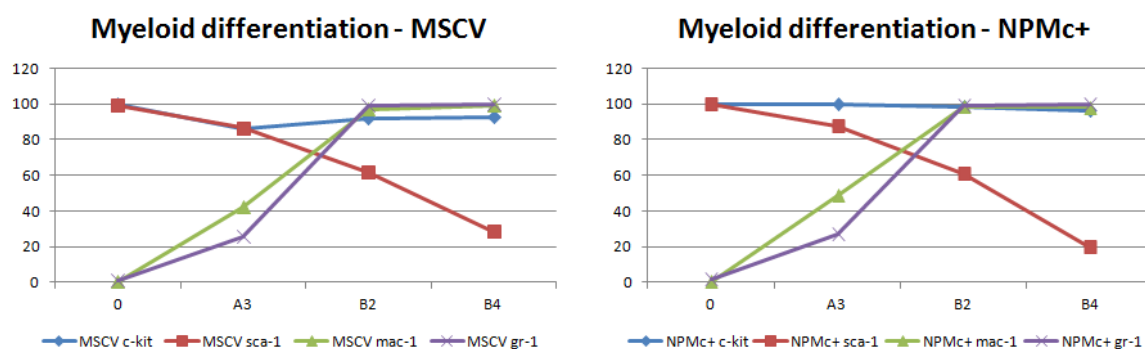


**Figure 24. Proliferation analysis of NPMc+ expressing cells.** BrdU incorporation analysis for NPMc+ and MSCV cells. Representative plots are depicted in the upper part of the figure. Percentage of cells in different cell cycle phases are reported in the table. One experiment out of three is reported.

### 2.1.2. Myeloid differentiation ability of NPMc+ expressing cell line

Next, we assessed the myeloid differentiation ability of NPMc+ expressing cells versus control cells. Differentiation of EML-C1 cells was detected by the decrease of stemness markers, c-kit and sca-1, and the increase in the percentage of cells expressing myeloid markers, namely mac-1 (also known as CD11b) and gr-1 after cytokine treatment. MSCV cells showed a mild increase of the myeloid markers at day

A3 (third day of medium A). In the second medium cells in differentiation were selected with the rapid increase of the percentage of cells expressing mac-1 and gr-1 (about 95-100%). c-kit remained high (80-100%) while sca-1 expressing cells decreased to 40-30%. NPMc+ expressing cells showed a very similar trend and no significant differences were reported in the percentage of positivity for four analyzed markers. In figure 25, an example of FACS results of cells in differentiation is reported: cells were monitored along the differentiation process and sample for FACS analysis were kept at the end of the incubation in the first medium with atRA (A3) and during the GM-CSF – based selection (B2 and B4). At each time point, cells were incubated with primary antibody conjugated with fluorochromes in order to identify the percentages of the cell population expressing c-kit, sca-1, mac-1 and gr-1, which are depicted in blue, red, green and purple in figure 25.

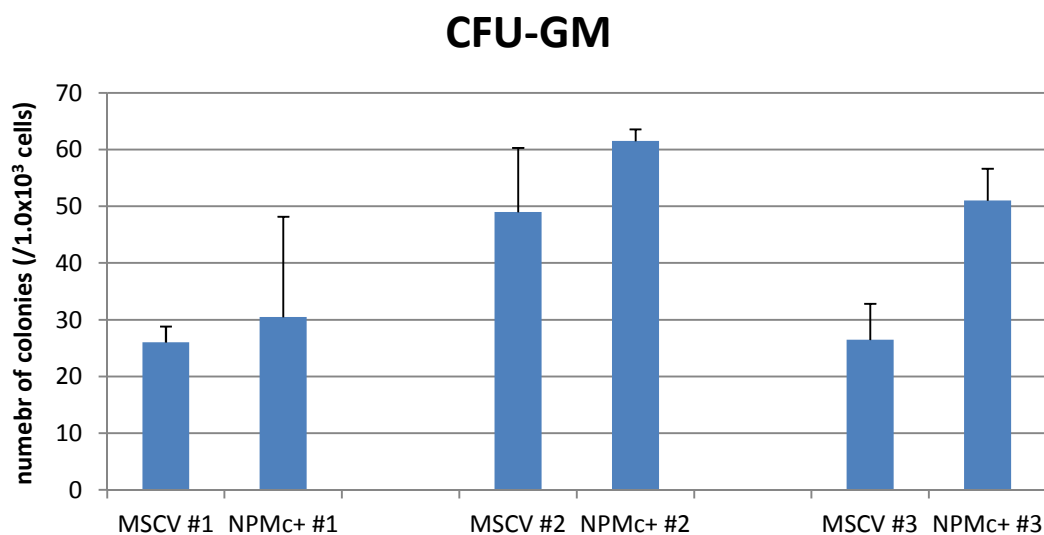


**Figure 25. FACS analysis of myeloid differentiation of MSCV and NPMc+ cells.** c-kit, sca-1, mac-1 and gr-1 were analyzed at the beginning of differentiation and during the differentiation protocol. A3 corresponds to the third day in medium A, B2 and B4 to the second and fourth day in medium B. The Y axis represents the percentage of positive cells for each surface marker.

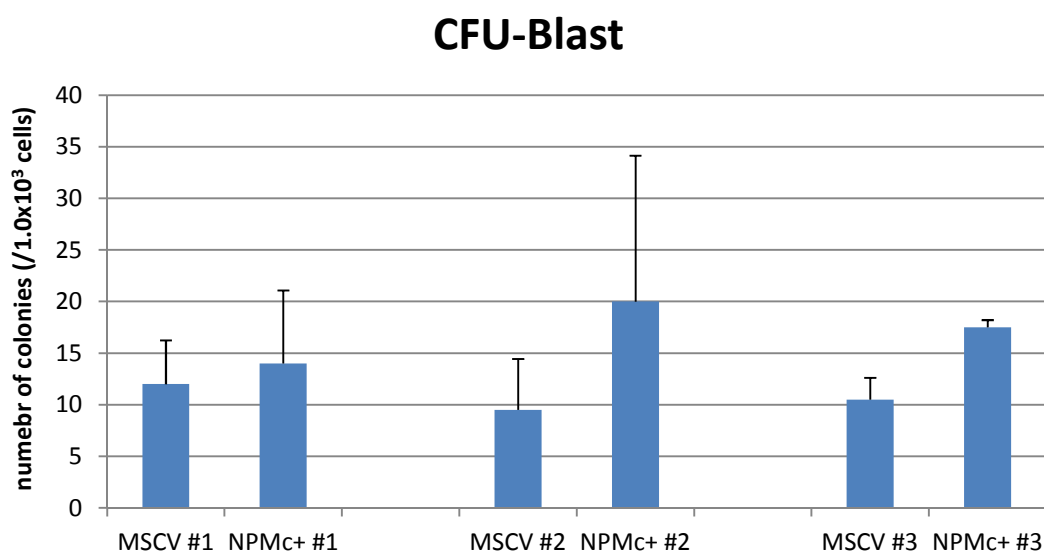
### 2.1.3. Clonogenic efficiency of NPMc+ expressing cells

In order to further characterize the NPMc+ expressing cell line, we performed a clonogenic assay. Cells were grown in methylcellulose medium in the presence of SCF and IL-3 for granulocyte-macrophage colony-forming units (CFU-GM corresponding to committed progenitors) or SCF alone for blast colony-forming units (CFU-Blast - corresponding to more primitive progenitors) [131]. Colonies were counted after 7

and 14 days, respectively. Results showed a slight increase in the number of CFU-GM colonies of NPMc+ expressing cells compared to MSCV. The same positive trend was detected in the number of CFU-Blast but in both cases the increase was not statistically significant (Figures 26 and 27 ).



**Figure 26. Clonogenic assay.** MSCV and NPMc+ were maintained in methylcellulose medium containing SCF and IL-3 for 7 days. Number of colonies was counted using ImageJ software.

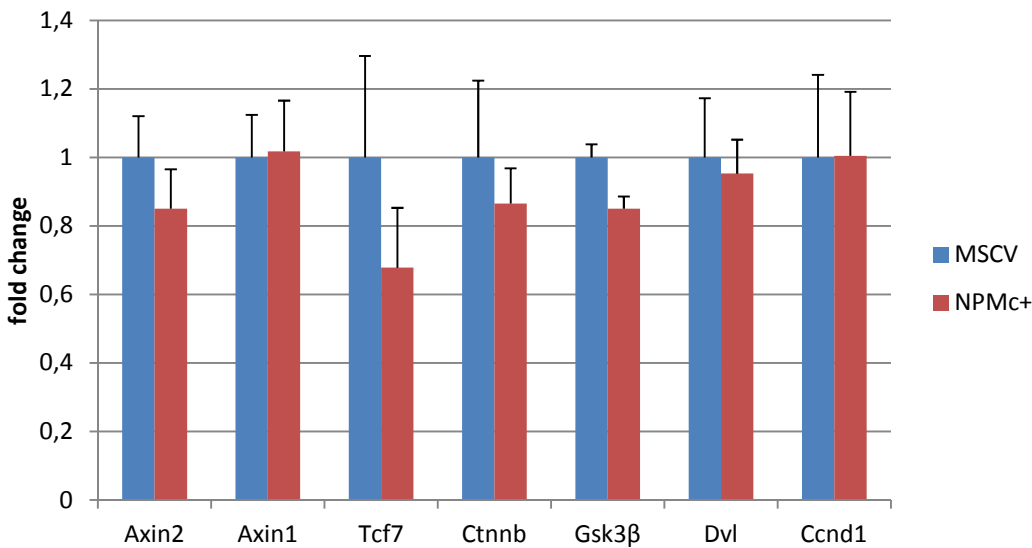


**Figure 27. Clonogenic assay.** MSCV and NPMc+ were maintained in methylcellulose medium containing SCF for 14 days. Number of colonies was counted using ImageJ software.

Taken together, our results suggest that expression of NPMc+ does not affect either proliferation or myeloid differentiation of EML-C1 cells.

#### 2.1.4. Wnt genes and Hox genes expression levels in NPMc+ cell line

In order to study the effect of NPMc+ on the activation of Wnt signaling in our *in vitro* system, quantitative RT-PCR analysis of Wnt target genes was performed. We focused our attention on genes involved in the pathway (*Gsk3 $\beta$* , *Dvl* and *Ctnnb* [63]) and genes that are direct targets of Wnt in other systems (*Ccnd1* [132]) or both (*Tcf7* [133], *Axin1/2* [134]).



**Figure 28. Expression levels of Wnt target genes and Wnt pathway component.** Quantitative PCR analysis were performed to assess the expression levels of canonical Wnt target genes and Wnt pathways components at steady state. TBP was used as normalizer.

As shown in the graph of figure 28, analyzed genes did not show an altered expression level in NPMc+ expressing cells compared to control. We concluded that NPMc+ expression in EML-C1 cells did not alter the Wnt target genes at steady state.

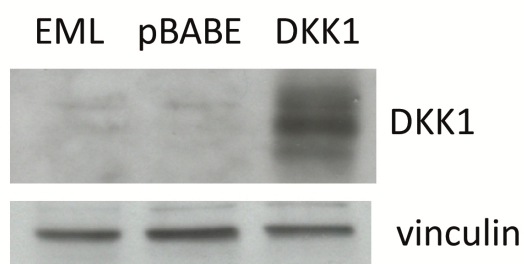
One of the characteristics features of NPMc+ AML is the upregulation of Hox genes, in particular of group A and B [40]. In order to study the effect of NPMc+ expression on the level of Hox genes in EML-derived cell line, we performed a quantitative RT-PCR. We chose to analyse those Hox genes that were reported as upregulated in Alcalay et



al. [40], namely *HoxA1*, *HoxA4*, *HoxA5*, *HoxA6*, *HoxA7*, *HoxA9*, *HoxA10*, *HoxB2*, *HoxB5*, *HoxB6*, *HoxB9*. Levels of analyzed Hox genes was undetectable both in the NPMc+ expressing and control cells.

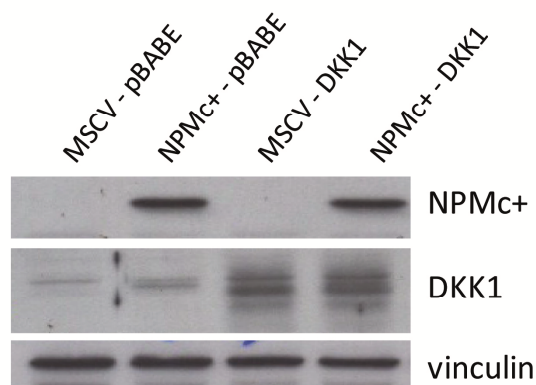
## 2.2. Generation and characterization of a cell line expressing both NPMc+ and Dkk1

A possible explanation for the lack of modulation of Wnt genes in NPMc+ cells is that Wnt signaling may be constitutively active in EML-C1 cells, and further activation by NPMc+ may therefore be irrelevant. To better investigate the effect of inhibition of Wnt signaling in EML-C1 cells, in particular of the canonical pathway, and the relationship between DKK1 and NPMc+ that was observed in zebrafish development and primitive hematopoiesis, we generated a double infected EML-C1 cell line carrying NPMc+ and overexpressing DKK1. Total RNA of p53<sup>-/-</sup> MEFs was reverse transcribed to cDNA, amplified by PCR and cloned into the pBABE retroviral vector to obtain the pBABE-DKK1 vector. pBABE and pBABE-DKK1 vectors were used to infect EML-C1 cells: as shown in the blot in figure 29, after puromycin selection, the bulk of infected cells overexpressed DKK1.



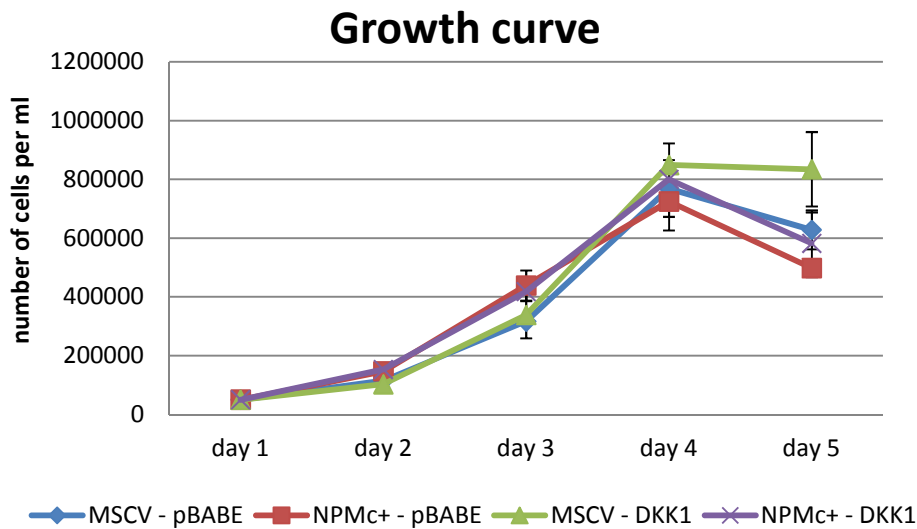
**Figure 29. Expression of DKK1 in pBABE-DKK1 infected EML-C1 cells.** Western blot showed the expression levels of DKK1 in the population of infected cells. pBABE cells were used as negative control. Vinculin was used as loading control.

The DKK1 cell line was then infected with MSCV-IRES-GFP and MSCV-IRES-GFP-NPMc+ vectors and sorted in order to generate double transfected cell line expressing NPMc+ and DKK1. In figure 30 a western blot is reported showing the expression level of NPMc+ and DKK1 in the NPMc+ - DKK1 cell line.



**Figure 30. Expression of NPMc+ and DKK1 in double infected cells.** Western blot showed the expression levels of NPMc+ and DKK1 in the population of double infected cell lines. pBABE-MSCV cells were used as negative control. Vinculin was used as loading control.

Despite the good expression of both NPMc+ and DKK1, as shown by the western blot, the cell lines did not differ massively from the controls for proliferation ability: in figure 31, an example of growth curve for NPMc+ DKK1 expressing cells and control cell lines (empty vector) is reported.



**Figure 31. Growth curve of NPMc+ and DKK1 expressing cells compared to controls.** MSCV - pBABE, NPMc+ - pBABE, MSCV - DKK1 and NPMc+ - DKK1 cells were seeded at 50000 cells/ml and counted each day. The Y axis represents the number of cells per ml of medium.

The four cell lines were monitored also for their myeloid differentiation ability: either overexpression of DKK1 alone or with NPMc+ did not alter the myeloid differentiation ability of cells, as detected by FACS analysis of surface markers (data not shown).

We expected an effect on the proliferative capacity of EML-C1 cells by overexpressing DKK1, an inhibitor of the canonical Wnt pathway, which is indeed known to be important for cell proliferation. The lack of any effect of DKK1 overexpression on EML-C1 cell line could be attributed to the characteristics of DKK1 itself and the way of action on the pathway: DKK1 is a diffusible molecule in the medium. EML-C1 cells are rapidly growing cells and the growing medium has to be changed very often, therefore the concentration of DKK1 in the medium may never reach an effective threshold to exert an effect. In order to study the effect of Wnt inactivation on EML-C1 cells and NPMc+ expressing cells we decided to take advantage of chemical inhibitors, as reported in section 2.4.

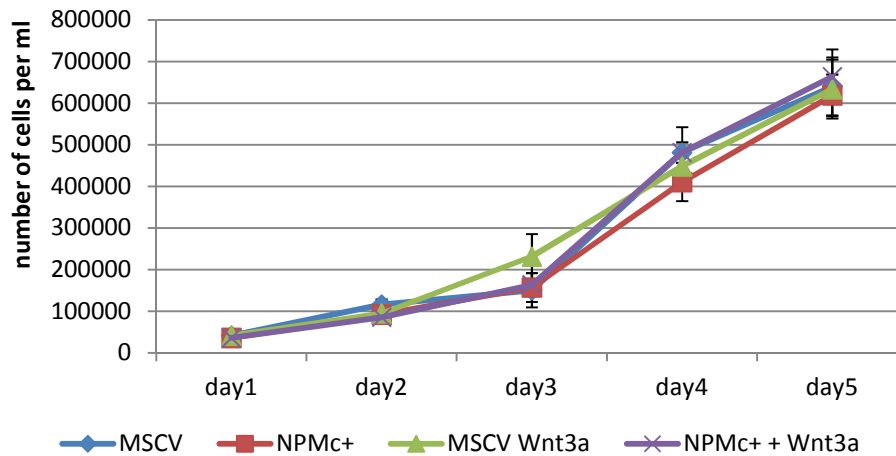
### 2.3. Response of NPMc+ expressing cells to Wnt signaling activation

In the previous sections, we described cell lines which highly expressed NPMc+ without showing any apparent change in their behavior, namely their proliferation and differentiation abilities. Moreover, we did not detect any effect of NPMc+ expression on Wnt signaling in the described experimental conditions. Next, we verified whether the effect of NPMc+ on Wnt signaling might be apparent only when the pathway is at least partly active, assuming that NPMc+ might not be able to activate the pathway by itself, but might potentiate other activators.

To this aim, chemical compounds and recombinant Wnt proteins are available. Considering that chemical compounds such as 6-bromoindirubin-3'-oxime (BIO) are used in literature but are not Wnt specific, we analyzed the response of control EML-C1 cells and NPMc+ expressing cells to the addition of two murine recombinant Wnt ligands, Wnt3a and Wnt5a in the growth medium. Wnt3a activates the canonical Wnt pathway, which leads to the enhancement of the unphosphorylated (active)  $\beta$ -catenin and the expression of Wnt target genes, such as *Ccnd1* and *Axin2*. Wnt5a acts on both the planar cell polarity (PCP) and the Wnt-Ca<sup>2+</sup> non-canonical pathways.

Cells were grown in presence and absence of either Wnt3a and Wnt5a and monitored every 24 hours in order to analyze the effect of Wnt activation on cellular proliferation. The number of cells counted was plotted in the graph in figure 32: MSCV cells do not show any difference in their proliferation in the presence of Wnt3a (green) compared to untreated cells (blue), as happens for NPMc+ expressing cells without (red) or in the presence of Wnt3a (purple). Wnt3a incubation does not have any effect on NPMc+ expressing cells proliferation when compared to control.

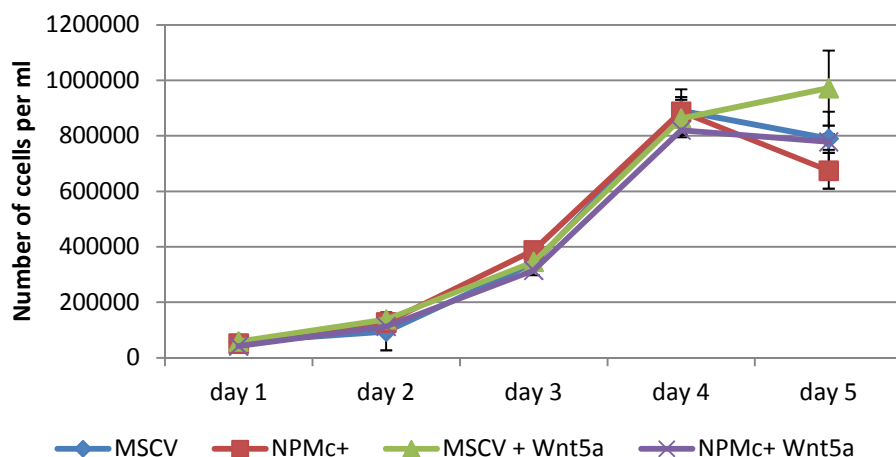
## Growth curve



**Figure 32. Growth curve of MSCV and NPMc+ expressing cells treated with Wnt3a.** NPMc+ and MSCV cells were seeded at 50000 cells/ml in Wnt3a containing medium (500 ng/ml) and counted each day. The number of NPMc+ (purple line) and MSCV (green line) cells per ml per day is reported. Untreated cells (red and blue lines) were used as control.

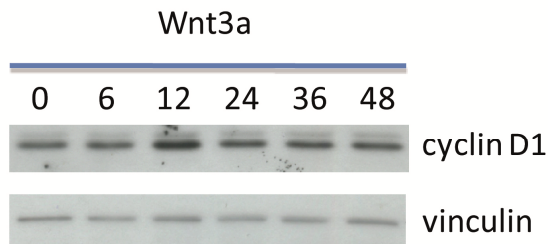
MSCV and NPMc+ cells were incubated with Wnt5a for 5 days and counted each day to analyze the proliferation ability of NPMc+ expressing cells in the presence of the ligand responsible for the activation of non canonical pathway. The presence of Wnt5a in the growth medium did not have effect on cellular proliferation (figure 33): proliferation of Wnt5a treated MSCV cells and NPMc+ cells (green and purple lines, respectively) was similar to not treated cells (blue and red lines, respectively). Therefore, no difference of proliferation ability was detected between Wnt5a - treated NPMc+ expressing cells (purple line) and control (green line).

## Growth curve

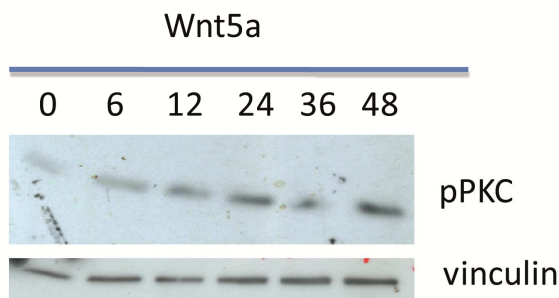


**Figure 33. Growth curve of MSCV and NPMc+ expressing cells treated with Wnt5a.** NPMc+ and MSCV cells were seeded at 50000 cells/ml in Wnt5a containing medium (500 ng/ml) and counted each day. The number of NPMc+ (purple line) and MSCV (green line) cells per ml per day is reported. Untreated cells (red and blue lines) were used as control.

In order to study the effect of Wnt3a and Wnt5a on the myeloid differentiation ability of NPMc+ expressing and control cells, we treated cells with the highest amount of ligands that did not result in toxicity before the induction of differentiation. We performed experiments on non-infected EML cells in order to identify the duration of Wnt treatment that causes a maximal response in cells. To measure activation by Wnt3a, the best readout would be the level of active  $\beta$ -catenin. Unfortunately, we had technical problems with the antibody, so we measured the levels of cyclin D1, a direct target of the canonical pathway. To assess Wnt5a activity, we analyzed the levels of phosphorylation of Protein Kinase C (PKC), a downstream effector of the PCP pathway. EML-C1 cells were incubated with 500 ng/ml of Wnt3a or 500 ng/ml of Wnt5a for 48 hours and samples for protein extraction and western blot were kept. The stronger response to Wnt3a was obtained after 12 hours of incubation, as shown by the blot against CyclinD1 in figure 34, while an incubation of 48 hours is necessary to see an increase in the phosphorylation of PKC (figure 35).



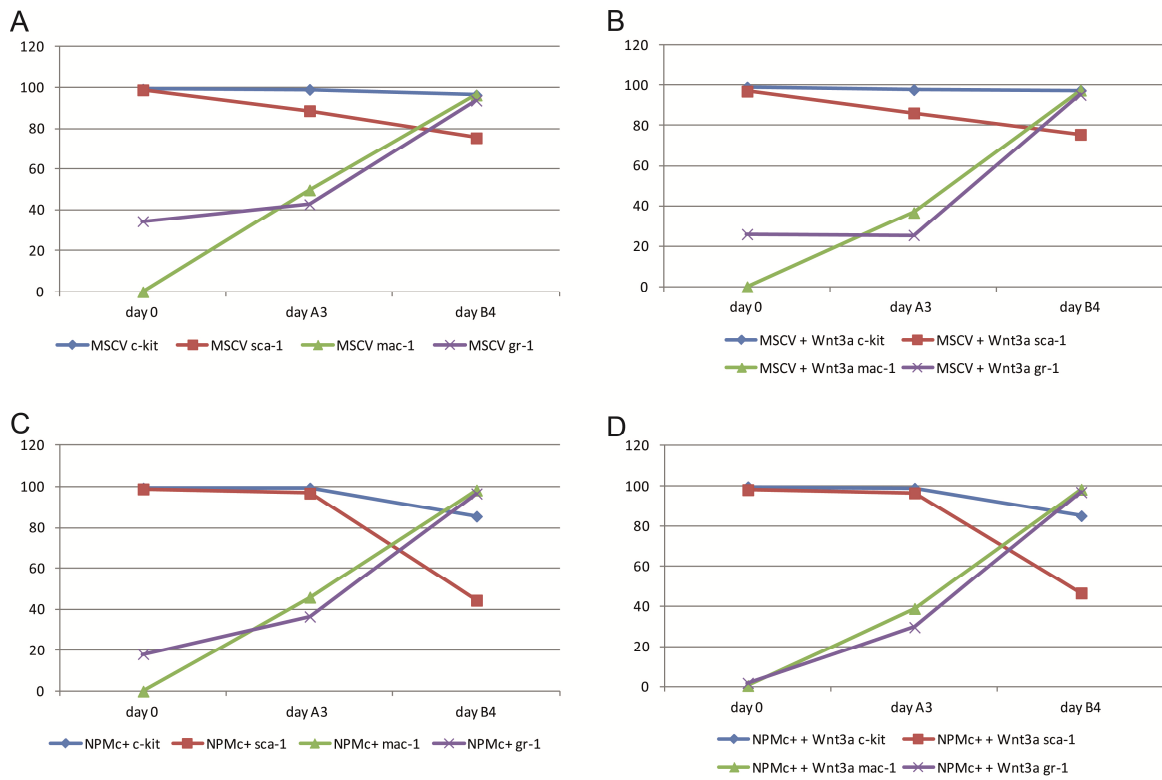
**Figure 34. Expression of Cyclin D1 in EML-C1 cells upon Wnt3a stimulation.** Western blot showed the expression levels of Cyclin D1 at different time points after addition of Wnt3a in growth medium. Vinculin was used as loading control.



**Figure 35. Expression of phosphorylated PKC in EML-C1 cells upon Wnt5a stimulation.** Western blot showed the expression levels of phosphorylated PKC at different time points after the addition of Wnt5a to the growth medium. Vinculin was used as loading control.

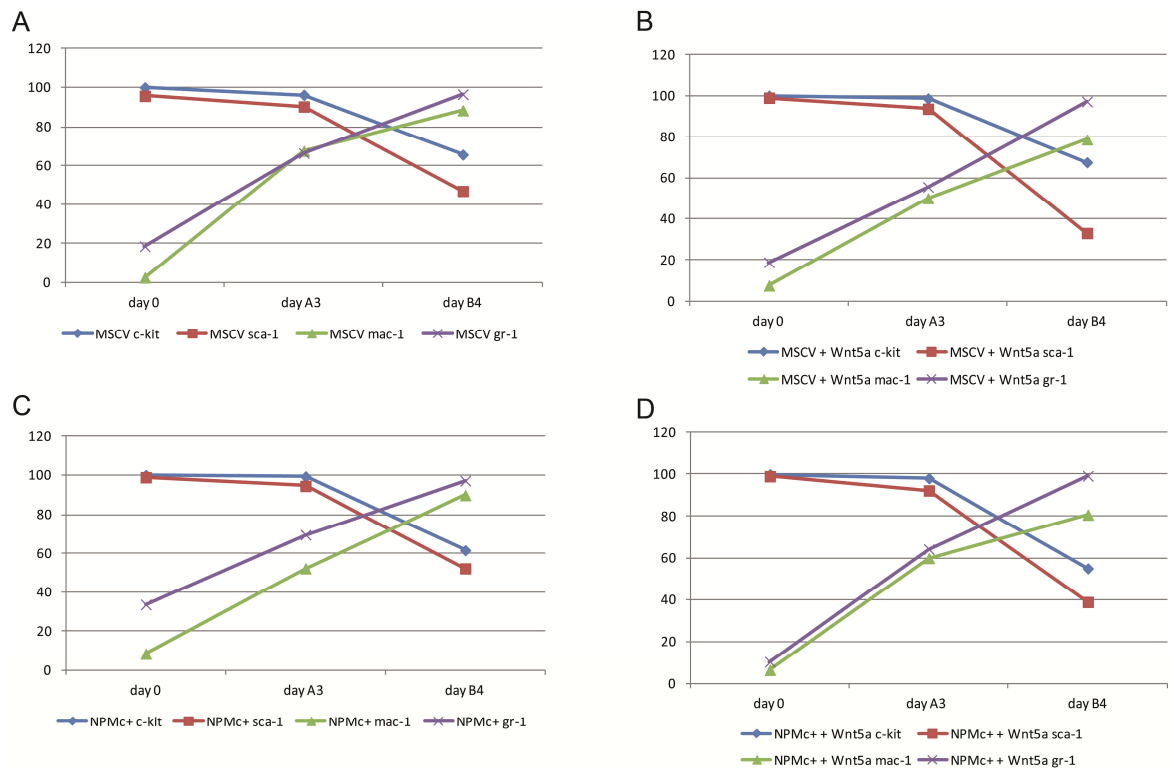
MSCV and NPMc<sup>+</sup> cells were then incubated with either Wnt3a for 12 hours or Wnt5a for 48 hours prior to induction of myeloid differentiation. Expression of surface markers was analysed by FACS at the beginning of the induction of differentiation and during the differentiation process, as already described in 2.1.2 section of “Results”. The percentage of cells expressing c-kit, sca1, mac-1 and gr-1 were very similar between MSCV and NPMc<sup>+</sup> cells, as already described in the previous section of the results. We did not detect any response of NPMc<sup>+</sup> expressing cells regarding their myeloid differentiation potential in the presence of Wnt ligands, Wnt3a and Wnt5a, compared to untreated NPMc<sup>+</sup> cells and to treated control cells (MSCV), as reported in figure 36 and 37.

We concluded that incubation with either Wnt3a and Wnt5a did not alter the behavior of EML-C1 cells, either expressing NPMc<sup>+</sup> or not.



**Figure 36. FACS analysis of myeloid differentiation of MSCV and NPMc+ cells treated with Wnt3a.** c-kit, sca-1, mac-1 and gr-1 were analyzed at the beginning of differentiation and during the differentiation protocol. A3 corresponds to the third day in medium A, B4 to the fourth day in medium B. The Y axis represents the percentage of positive cells for each surface marker. A: untreated MSCV cells; B: Wnt3a treated MSCV cells (500 ng/ml); C: untreated NPMc+ cells; D: Wnt3a treated NPMc+ cells (500 ng/ml).





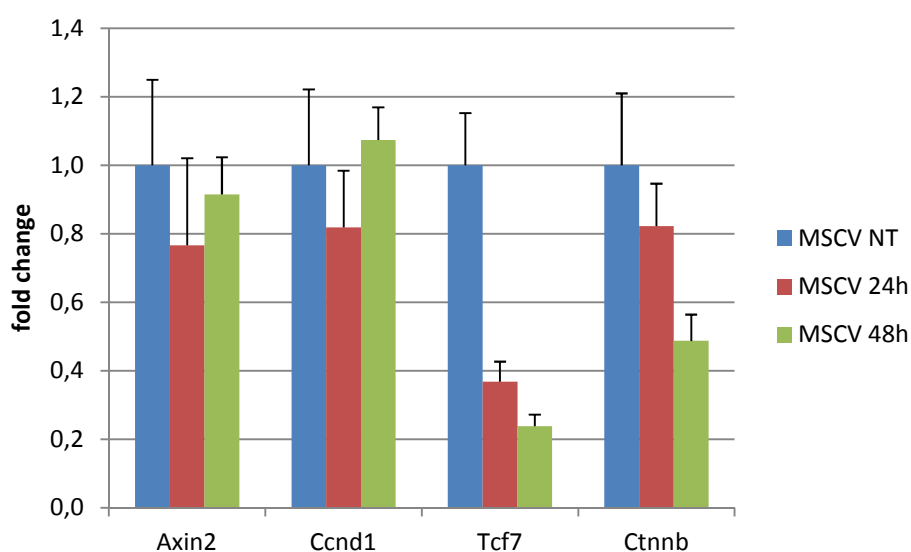
**Figure 37. FACS analysis of myeloid differentiation of MSCV and NPMc+ cells treated with Wnt5a.** c-kit, sca-1, mac-1 and gr-1 were analyzed at the beginning of differentiation and during the differentiation protocol. A3 corresponds to the third day in medium A, B4 to the fourth day in medium B. The Y axis represents the percentage of positive cells for each surface marker. A: untreated MSCV cells; B: Wnt5a treated MSCV cells (500 ng/ml); C: untreated NPMc+ cells; D: Wnt5a treated NPMc+ cells (500 ng/ml).

## 2.4. Response of NPMc+ expressing cells to indomethacin treatment

The positive modulation of Wnt signaling by NPMc+ seen in the zebrafish could be indirect, namely due to a lack of or a decreased response to Wnt inhibition. To study the response of NPMc+ expressing cells to Wnt inhibition, we treated cells with indomethacin, a chemical inhibitor of Wnt signaling. Indomethacin has been used to inhibit the canonical pathway as it was shown to indirectly decrease the protein level of  $\beta$ -catenin, thus affecting the downstream transcriptional program. We treated cells with increasing amounts of indomethacin for 48 hours in order to obtain the concentration which triggers an effect without affecting cellular viability. Control cells

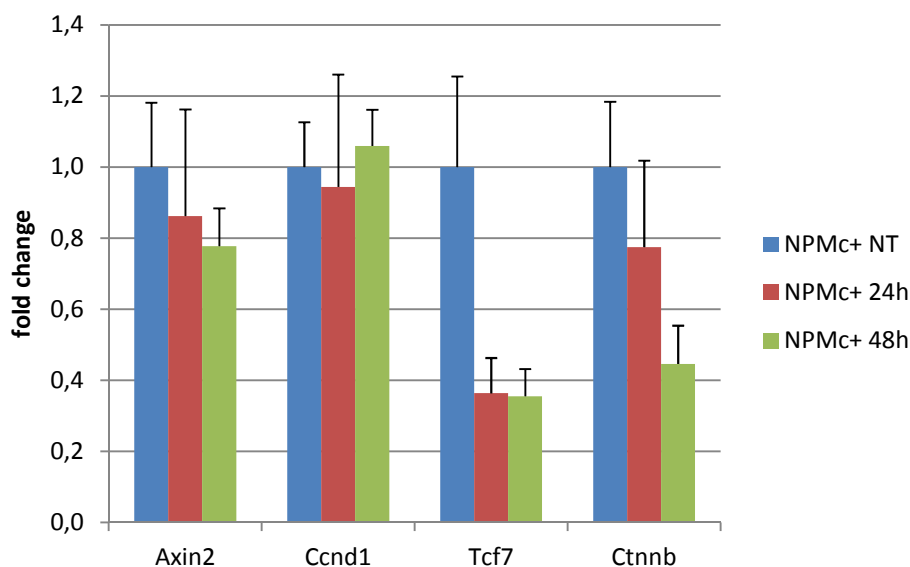
were incubated with a concentration of indomethacin ranging from 100  $\mu$ M up to 1mM. After 24 hours, cells treated with 600  $\mu$ M or higher concentrations of indomethacin shown signs of apoptosis and no live cells were detected at 48 hours. We therefore choose to treat NPMc+ expressing cells with 500  $\mu$ m of indomethacin and we analyzed the expression level of Wnt genes (*Cttnb*, *Axin2*, *Tcf7*, *Ccnd1*) 24 and 48 hours of treatment.

Control cells did not respond massively to indomethacin treatment, as shown by the modest decrease in expression of *Axin2* and *Ccnd1* (encoding Cyclin D1) in MSCV cells. The effect of Wnt inhibitor is stronger on *Cttnb* (encoding  $\beta$ -catenin) and *Tcf7* expression, which after 24 hours of indomethacin treatment decreased respectively by approximately 20% and 60%. After 48 of treatment, *Ccnd1* and *Axin2* levels returned similar to untreated cells, while *Cttnb* and *Tcf7* were still downregulated. A very similar trend was observed in NPMc+ expressing cells. *Axin2* and *Ccnd1* expression levels were unchanged after 24 hours and 48 hours. *Tcf7* and *Cttnb* were downregulated after both 24 and 48 hours of treatment at levels comparable to those of control cells (MSCV). In figure 38 and 39, results of one representative experiment out of 4 are reported.



**Figure 38. MSCV cells response to indomethacin treatment.** MSCV cells were incubated in EML growth medium supplemented with 0.5  $\mu$ M of indomethacin for 24 and 48 hours. Cells were harvested

and expression levels of *Axin2*, *Ccnd1*, *Tcf7* and *Ctnnb* were analyzed by qPCR and compared to untreated cells. *Tbp* was used as normalizer.



**Figure 39. NPMc+ cells response to indomethacin treatment.** NPMc+ cells were incubated in EML growth medium supplemented with 0.5  $\mu$ M of indomethacin for 24 and 48 hours. Cells were harvested and expression levels of *Axin2*, *Ccnd1*, *Tcf7* and *Ctnnb* were analyzed by qPCR and compared to untreated cells. *Tbp* was used as normalizer.

Taken together our results suggest that expression of NPMc+ in EML-C1 cells does not result in modulation of Wnt signaling.

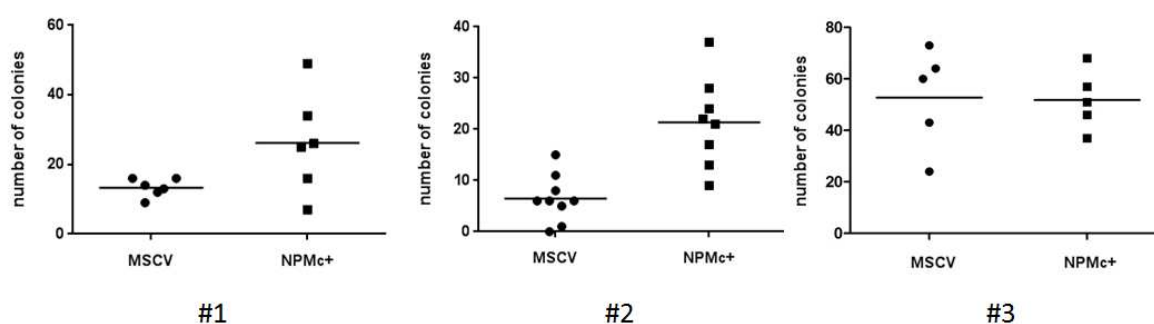
## 2.5. Stroma interaction

In the bone marrow, hematopoietic stem cells are physiologically surrounded by a plethora of cells that form the hematopoietic niche. These cells are responsible for the homeostasis of HSCs by releasing molecules that are necessary for the maintenance of stem cells characteristics. In turn, cells within the niche receive signals for the niche behavior.

*In vitro* studies limit the analysis of the effect of the microenvironment on the behavior of hematopoietic stem and progenitor cells, in terms of proliferation, differentiation and gene expression program. To mimic the presence of the microenvironment, cells can be grown in the presence of recombinant proteins that

are known to be released in the niche, or grown in contact with primary stroma or on a layer of a stroma-derived cell line.

We studied whether the interaction with the microenvironment might be important for NPMc+ functions. To mimic the complexity found in the bone marrow microenvironment and HSC niche, we performed a LTC-IC assay. NPMc+ expressing EML-C1 cells and control cells were maintained in contact with primary stroma derived from bone marrow cells of C57/B6 mice for 5 weeks, then diluted 1 to 10 and grown on methylcellulose medium. After one week the number of colonies derived from NPMc+ expressing and control cells were counted.



**Figure 40. Long-term culture-initiating cell assay.** Number of colonies of MSCV and NPMc+ grown in methylcellulose medium after prolonged interaction with murine primary BM stroma.  $1.0 \times 10^4$  cells/well were seeded on irradiated primary stroma in a 6-well plate.

In two out of three experiments, total number of colonies of NPMc+ expressing cells was higher than control derived colonies (figure 40, experiments 1 and 2); however, only in one case the difference was statistically significant (figure 40, experiment 2 p-value<0,05).

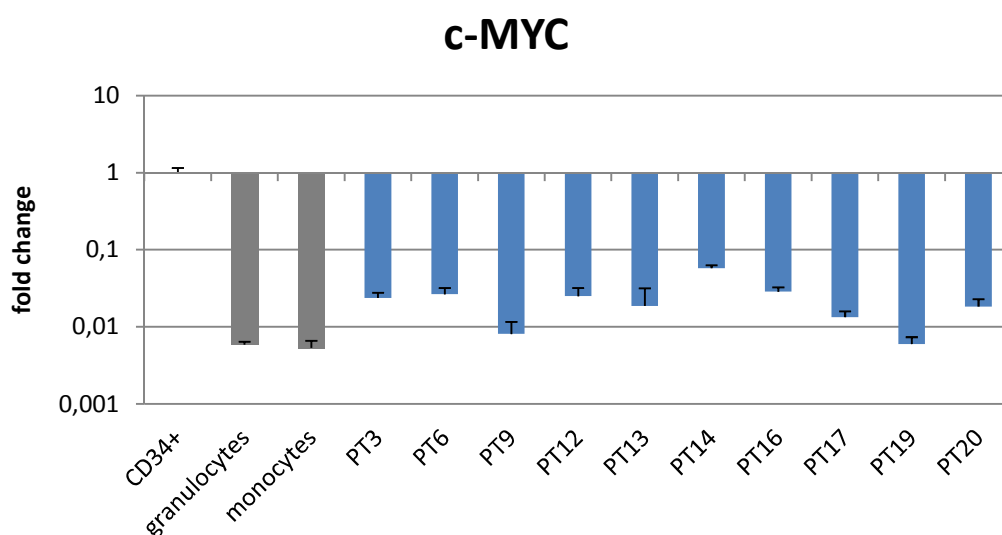
### 3. Wnt pathway in NPMc+ AML patients

As described in the introduction, activation of Wnt signaling has been described in different subtypes of leukemias, but not for NPMc+ AML. As reported in the first

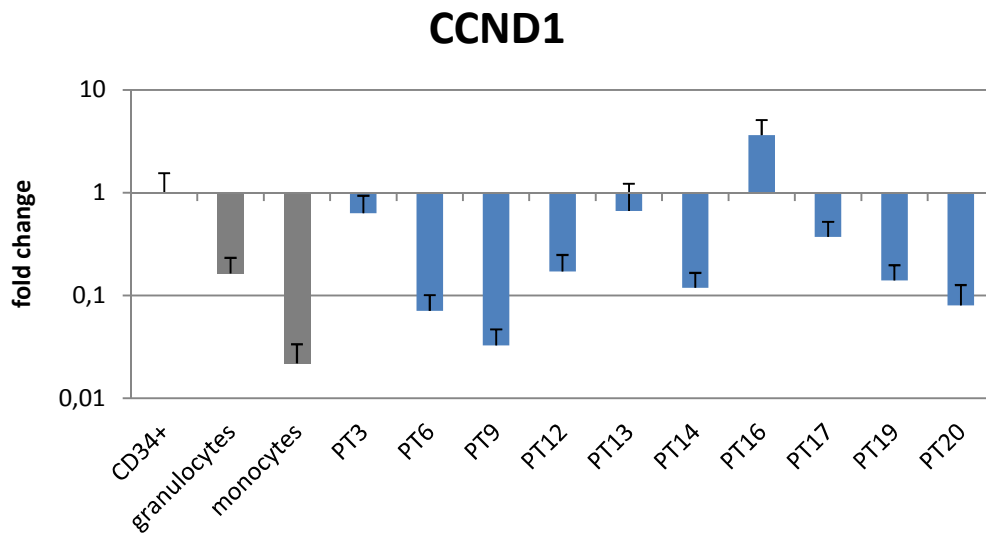
part of the “Results” section, we found a connection between NPMc+ and Wnt signaling during zebrafish early development and primitive hematopoiesis. The attempts to investigate the positive modulation of Wnt signaling by NPMc+ in an established murine cell line, namely EML-C1, were not successful. We then decided to analyze the activation of Wnt signaling in blasts from NPMc+ AML patients.

The first challenge was the choice of the appropriate control. Wnt signaling is indeed modulated by the expression of other leukemogenic oncoproteins [100], thus, the comparison of NPMc+ AMLs versus other AMLs would not give reliable results. For these reasons, we did not compare the level of Wnt target genes in NPMc+ AML samples to other AML samples but to normal human CD34+ cells, granulocytes and monocytes isolated from peripheral blood of healthy donors.

We decided to focus our attention on the canonical Wnt signaling to analyze the expression of well-known and widely activated target genes of this pathway: *c-MYC*, *CCND1* (CyclinD1). Patient RNA was retrotranscribed to cDNA and analysed through qPCR. Both *c-MYC* and *CCND1* are generally downregulated in NPMc+ AML patients (figure 41 and 42).

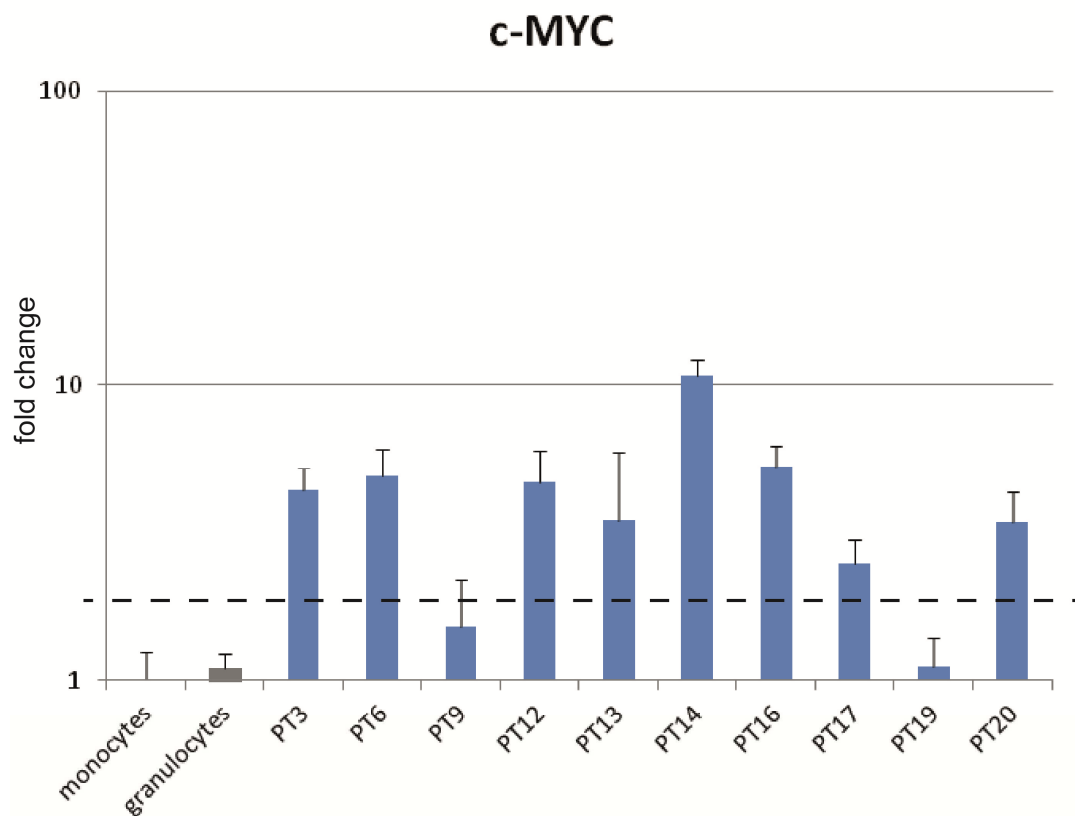


**Figure 41. *CCND1* expression in NPMc+ AML patients compared to CD34+ cells of healthy donors.** TATA binding protein (TBP) was used as normalizer.

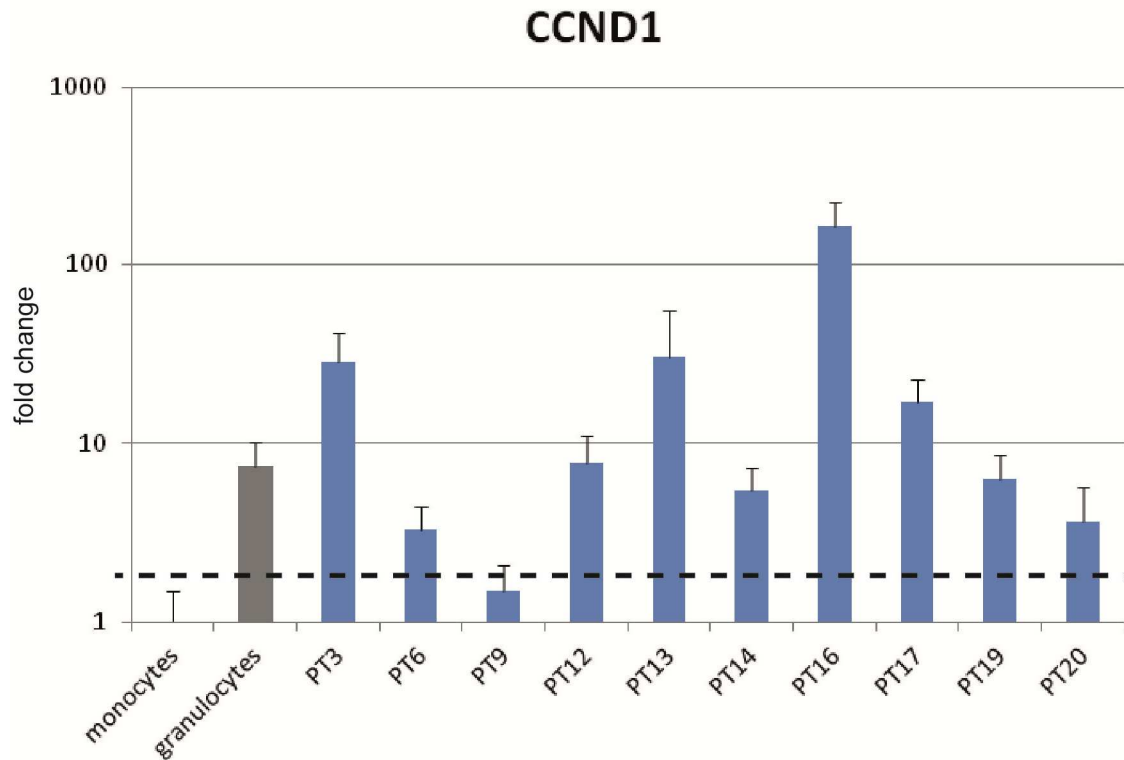


**Figure 42. *CCND1* expression in NPMc+ AML patients compared to CD34+ cells of healthy donors.** TBP was used as normalizer.

NPMc+ blasts are cells blocked along the myeloid lineage but NPMc+ AML cases are most frequent among the M4-M5 AML, characterized by more differentiated leukemic blasts. For this reason, we compared *c-MYC* and *CCND1* expression level in NPMc+ AML blasts to monocytes (CD14+ cells) of healthy donor. *c-MYC* was highly overexpressed in NPMc+ AML samples compared to CD14 positive cells (10 out of 13 samples > 2 fold change, figure 43); *CCND1* was overexpressed in 12 out of 13 NPMc+ samples (figure 44).



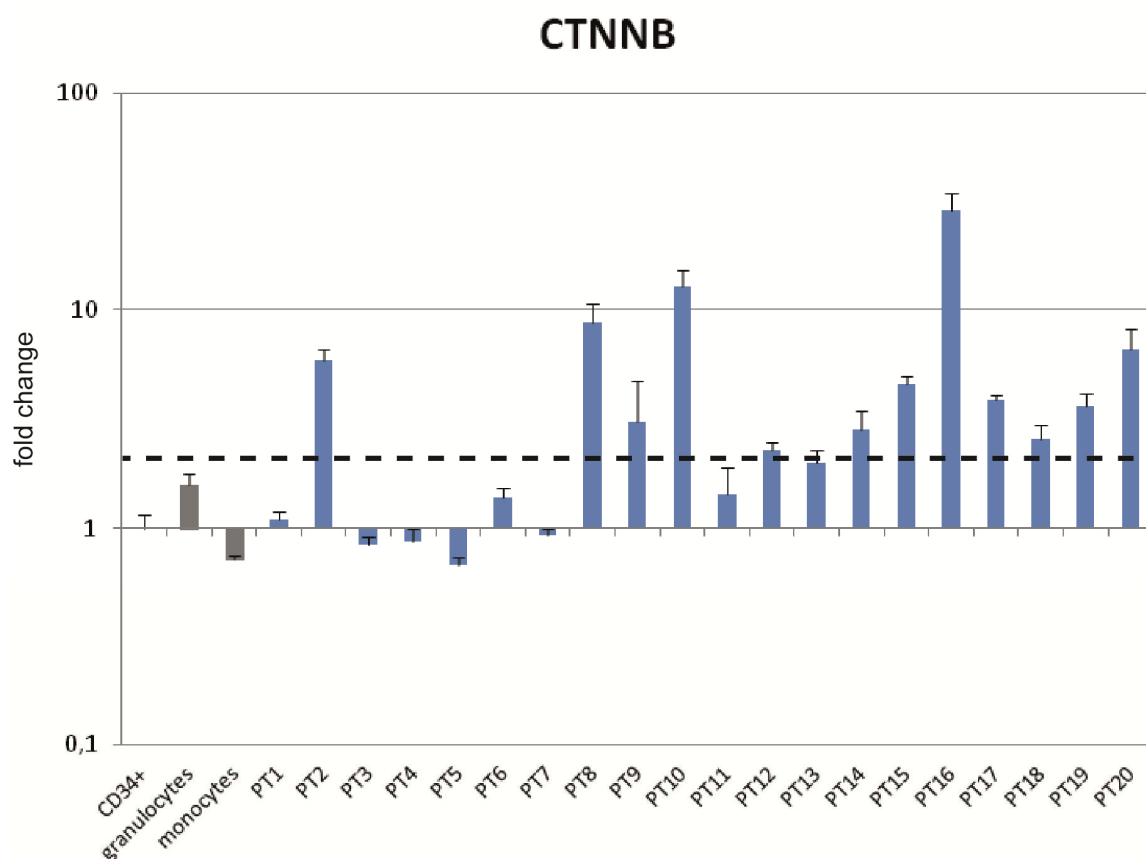
**Figure 43.** *cMYC* expression in NPMc+ AML patients compared to CD14+ cells of healthy donors. TBP was used as normalizer. Dashed line correspond to 2-fold change.



**Figure 44.** *CCND1* expression in NPMc+ AML patients compared to CD14+ cells of healthy donors. TBP was used as normalizer. Dashed line correspond to 2-fold change.

We then analyzed the expression levels of three component of Wnt pathway, *AXIN2*, *AXIN1* and *CTNNB* ( $\beta$ -catenin). *CTNNB* encodes  $\beta$ -catenin, the main effector of the canonical Wnt pathway. *AXIN1* and *AXIN2* are components of the destruction complex, which controls the cytoplasmic concentration of  $\beta$ -catenin. Moreover, *AXIN2* is a target of the pathway through a negative feedback loop.

Expression levels of *CTNNB* were analysed in 20 patients' samples of NPMc+ AML: in 10 out of 20 samples an overexpression of *CTNNB* was detected (threshold of 2-fold), while 4 of them showed a lower expression of *CTNNB* compared to CD34+ cells (figure 45).

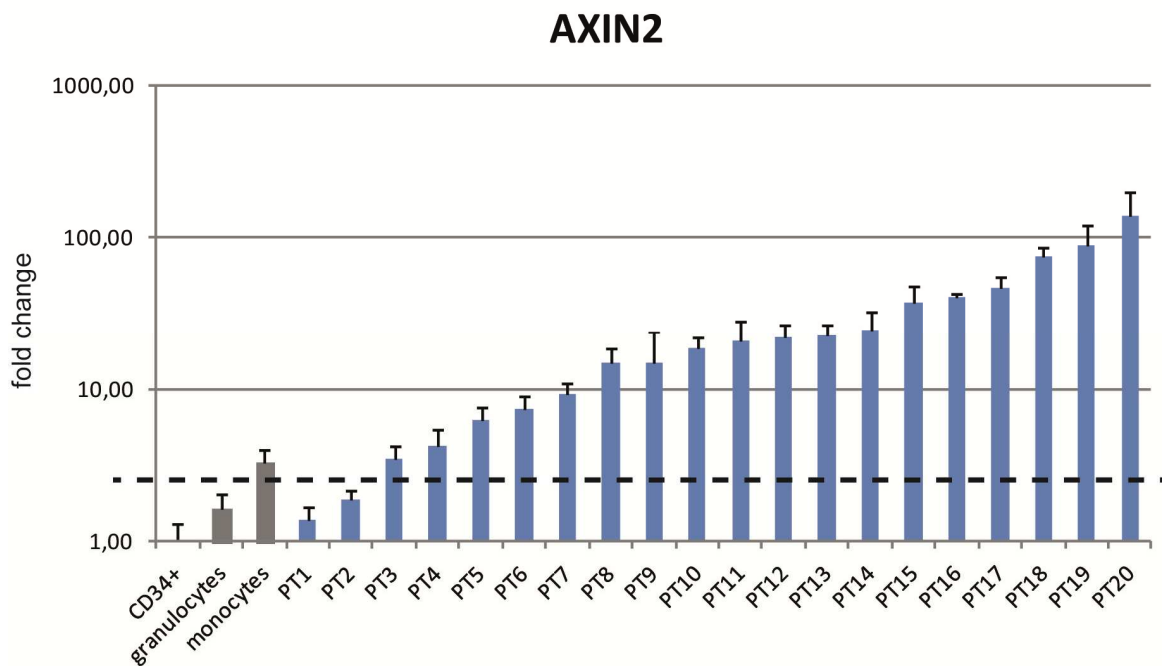


**Figure 45. *CTNNB* expression in NPMc+ AML patients compared to CD34+ cells of healthy donors.** TBP was used as normalizer. Dashed line correspond to 2-fold change.

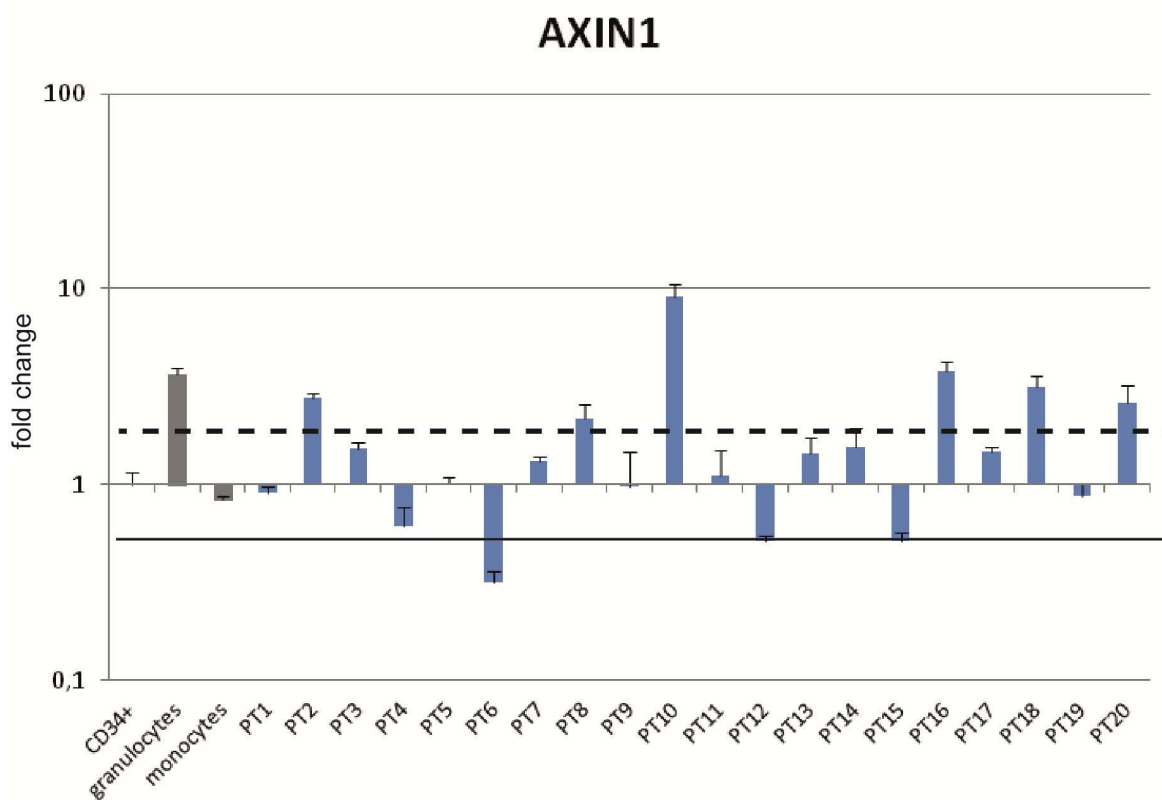
Comparing expression level of *AXIN1* and *AXIN2* in NPMc+ AML samples to CD34+ cells from healthy donors, we observed that *AXIN2* was generally overexpressed in NPMc+ blasts (figure 46). *AXIN1* was, instead, variably expressed (figure 47): 6 out of



20 samples presented an overexpression > 2-fold, while 2 patients showed a downregulation of *AXIN1* (< 0.5-fold compared to CD34+ cells).



**Figure 46. *AXIN2* expression in NPMc+ AML patients compared to CD34+ cells of healthy donors.** TBP was used as normalizer. Dashed line correspond to 2-fold change.



**Figure 47. *AXIN1* expression in NPMc+ AML patients compared to CD34+ cells of healthy donors.** TBP was used as normalizer. Dashed line correspond to 2-fold change. Black line corresponds to 0,5-fold change.

Taken together, our results showed that level of Wnt target genes are quite variable in individual samples, suggesting that Wnt signaling might be activated by NPMc+ in NPMc+ AML blasts.

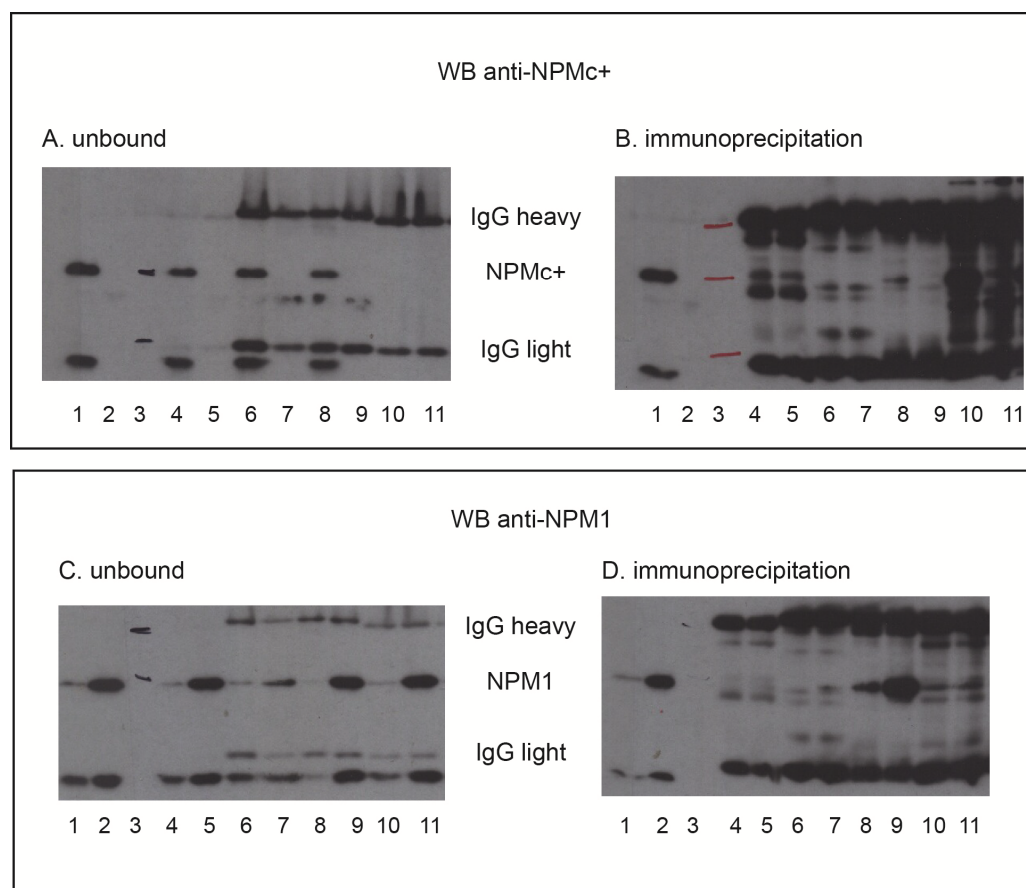
#### **4. Set up of immunoprecipitation protocol for endogenous NPM1 and NPMc+ in OCI-AML3 cell line**

The different cellular localization of NPM1 and its mutant NPMc+ suggests that the two proteins could interact with different partners. While NPM1 has been reported to interact with nucleic acids and play a role in maturation and stabilization of mRNAs [51, 135], this association has not yet shown for NPMc+. In order to identify NPMc+ specific interactors and to gain insight into the possible mechanism of NPM1 and NPMc+ modulation of Wnt signaling, we set up a protocol to immunoprecipitate either the wt or the mutant version of NPM1. The studies conducted so far to identify NPM1 interactors were based on its overexpression in non-hematopoietic cell lines, namely MEFs, U2OS and Hela cells [21, 22]. We decided to investigate the interactors of NPM1 and NPMc+ in a more physiological situation, immunoprecipitating either NPM1 or NPMc+ in the OCI-AML3, a patient derived cell line that carries the NPM1 mutation A in heterozygosity.

We took advantage of two antibodies that recognize the two proteins: a commercial antibody for the wild-type NPM1 and the T26 antibody, which was developed in our institute, for NPMc+ [124].

We tested the capability of the two antibody to immunoprecipitate their targets. The anti-NPMc+ antibody is able to precipitate NPMc+ from the OCI-AML3 cell line (figure 48B, lane 10), which carries mutation A, but does not specifically precipitate any protein of the same size in the OCI-AML2 cell line (48B, lane 11), which does not

carry the mutation. Unluckily, the antibody pulled down a great amount of debris or not specific interactions at this concentration (1:50 dilution). NPMc+ was undetectable in the unbound fraction (48A lane 10), suggesting that the majority of the NPMc+ pool was bound by the antibody in these experimental conditions.



- |                   |                       |                         |
|-------------------|-----------------------|-------------------------|
| 1. input OCI-AML3 | 4. anti-V5 OCI-AML3   | 8. anti-NPM1 OCI-AML3   |
| 2. input OCI-AML2 | 5. anti-V5 OCI-AML2   | 9. anti-NPM1 OCI-AML2   |
| 3. marker         | 6. anti-flag OCI-AML3 | 10. anti-NPMc+ OCI-AML3 |
|                   | 7. anti-flag OCI-AML2 | 11. anti-NPMc+ OCI-AML2 |

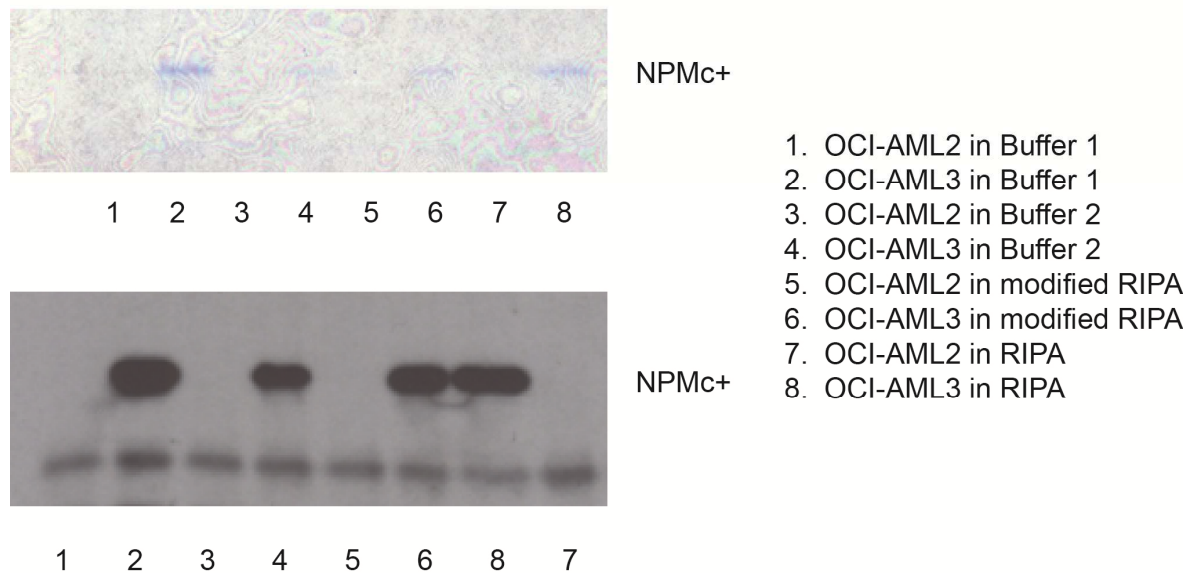
**Figure 48. Immunoprecipitation capacity of anti-NPM1 and anti-NPMc+ antibodies.** Anti-NPM1 and anti-NPMc+ antibodies were tested for their capacity to bind NPM1 and NPMc+. Antibodies were incubated in the presence of whole cell lysates of OCI-AML2 and OCI-AML3 cells and complexes were immunoprecipitated with sepharose beads. Both unbound (A, C) and immunoprecipitated fractions were loaded on a polyacrilamide gel and blotted with anti-NPMc+ (A-B) and anti-NPM1 (C-D) to reveal the presence of the proteins. Inputs correspond to 10% of the quantity of whole cell lysate used for each IP and unbound corresponds to 1/30 of the IP solutions. Anti-V5 and anti-flag were used as negative controls. OCI-AML2 was used as negative control in NPMc+ immunoprecipitation.

The commercial anti-NPM1 antibody was able to precipitate NPM1 from both OCI-AML2 (figure 48D, lane9) and OCI-AML3 (figure 48D, lane8) cells. A relevant amount of NPM1 remained in the unbound fraction of OCI-AML2 lysate (figure 48C, lane 9),

while NPM1 was undetectable in the unbound fraction of the OCI-AML3 lysate (figure 48C, lane 8).

We then assessed the cross reaction of the two antibodies. A faint band was detectable in figure 48B, lane 8, in correspondence of the IP for NPM1 blotted with NPMc+: the presence of the band would suggest a cross-reaction of the NPM1 antibody for NPMc+. Otherwise, it might be explained by the co-precipitation of a part of NPMc+ protein pool with NPM1, given the interaction between the two proteins. Similarly, we detected two faint bands in correspondence of the immunoprecipitation performed with NPMc+ antibody (figure 48D, lanes 10 and 11) which might be due either to cross reaction of the antibody or co-precipitation of NPM1 interacting with NPMc+.

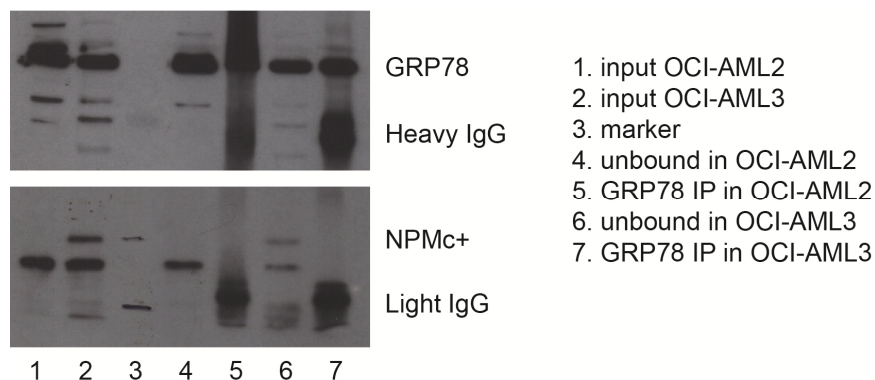
Next, we tested different lysis conditions to obtain the largest amount of protein while maintaining the interactions with peptidic and nucleotidic partners of NPM1 and NPMc+. Buffer 1, buffer 2, modified RIPA and RIPA buffers differ slightly for Tris and sodium chloride concentrations, presence and concentration of detergents (sodium dodecyl sulfate or NP-40), presence of DTT or sodium deoxycholate. 1 mg of lysates of OCI-AML2 and OCI-AML3 cells were incubated with 4 µg of anti-NPM1; the immunoprecipitated was then analyzed in a gel, stained with coomassie blue.



**Figure 49. Lysis Buffers test.** Different lysis conditions were tested in order to optimize the amount of immunoprecipitated protein. The highest amount of NPMc+ protein was detected in the line 2 in both the coomassie blue stained gel and the western blot, corresponding to the immunoprecipitation performed with Buffer 1.

In figure 49, the results of the immunoprecipitation of NPMc+ in 4 different buffers were reported. The highest quantity of NPMc+ protein was pulled down with buffer 1 and RIPA buffer, which indeed corresponded to the buffers that extracted the highest quantity of protein (western blot). We chose to continue the IP setting with Buffer 1, while RIPA buffer might be too strong to maintain the interactions within proteins. A preliminary experiment of immunoprecipitation followed by mass spectrometry was performed.

Most of the detected proteins are highly expressed and were probably false positives, suggesting that an increase in the stringency of the experimental conditions or in the whole protocol has to be made. In fact validation of one putative partner included in the mass spectrometry results, the 78 kDa Glucose-regulated protein (GRP78), by the reverse IP performed with a specific antibody recognizing did not revealed any interaction of GRP78 with NPMc+ (figure 50).



**Figure 50. Immunoprecipitation of GRP78.** Anti-GRP78 immunoprecipitation was blotted with anti-GRP78 and anti-NPMc+ antibodies. Inputs correspond to 10% of the quantity of whole cell lysate used for each IP and unbound correspond to 1/30 of the IP solutions.

We are currently optimizing the protocol in order to increase the specificity of protein-protein interactions. First, we will introduce a step of formaldehyde fixation of the OCI-AML2 and OCI-AML3 cell lines in order to stabilize interactions of NPM1 and NPMc+ with their respective partners. Furthermore, we will change the beads for the precipitation step, from sepharose beads to magnetic beads, which strongly decreased the non-specific binding., and we will scale up the starting amount of lysate, from 3-5 mg up to 10 mg or more.

## **DISCUSSION**





Leukemogenesis derives from an alteration of hematopoiesis, which normally leads from a pool of progenitors and stem cells (HSC) to the formation of all blood lineages. Homeostasis and maintenance of HSCs require their interaction with cellular components and signaling molecules of the hematopoietic niche. Cellular signaling pathways, such as Notch and Wnt, play a pivotal role in this process and their alteration may contribute to the leukemogenic process.

Bertrand et al. demonstrated that functional Notch signaling is dispensable for primitive hematopoiesis but it is required for definitive hematopoiesis [136, 137]. Notch signaling is important for the expression of hematopoietic markers like *Runx1* [137], *Gata2* [138] and *Tal1* [138]. Its role in adult hematopoiesis is less clear but it has been shown that Notch activation has the potential to increase the pool of multipotent progenitors [139, 140].

The role of Wnt signaling in hematopoiesis and leukemogenesis has been extensively studied. Interestingly, different approaches resulted in opposite conclusions: on one hand the expression of a constitutively active form of  $\beta$ -catenin in HSCs of Bcl2-transgenic mice led to an increase of their proliferation and repopulation abilities [85] while a stable form of  $\beta$ -catenin led to a transient expansion of the hematopoietic stem cell compartment and its subsequent exhaustion [89, 90].

As hypothesized some years ago by Scheller et al [90] and then demonstrated by Luis and colleagues [84], fine tuning of Wnt signaling is necessary for hematopoiesis and for the maintenance of hematopoietic stem cells. Alteration of Wnt signaling may

therefore result in an increase of HSC proliferation as well as in the block of hematopoiesis when the pathway is too strongly activated [84].

Wnt signaling is indeed active in AML: MLL-AF9 [102], AML1-ETO [141] and PML-RAR $\alpha$  [101] activate Wnt signaling, suggesting an involvement of this pathway in establishment and/or progression of the disease.

Connection with Wnt signaling has not yet been described in NPMc+ AML, a subtype of AML characterized by the cytoplasmic mislocalization of the nucleolar protein NPM1. We showed that both NPM1 and its leukemia-associated mutant, NPMc+, are able to modulate in opposite fashion Wnt signaling during embryonic development and to enhance the proliferation of hematopoietic progenitors in vertebrates. We also tried to recapitulate these findings in a mammalian in vitro system to dissect the mechanism underlying the modulation of Wnt by NPMc+.

### **NPMc+ expression leads to myeloproliferation**

Several models of NPMc+ AML have been generated in the attempt of elucidate the leukemogenic mechanism of the mutant protein; however its function is still not clearly understood. We took advantage of the accessibility and transparency of zebrafish embryos to analyze the effect of NPMc+ expression in zebrafish hematopoiesis, and in particular in the establishment of hematopoiesis in the early stages of development.

NPMc+ expression leads to an increase in the pool of hematopoietic precursors at early and late stages of primitive hematopoiesis, as shown by the increased expression of *gata2*, *lmo2* and *tal1* (fig. 16 and 17).

In primitive zebrafish hematopoiesis, hematopoietic progenitors can differentiate toward the myeloid and the erythroid lineages only. We found that NPMc+ expression is associated with an increase in the pool of myeloid precursors (fig. 18) that did not

result in the expansion of more differentiated cells (*l-plastin*, *mpx* expressing cells, fig. 19), suggesting that the proliferative effect of NPMc+ could be restricted to progenitors in primitive hematopoiesis.

We observed also a modest increase in the pool of erythroid cells when *NPMc+* mRNA was injected into embryos (fig.20).

Our results confirmed the effect described by Bolli and collaborators triggered by NPMc+ expression in zebrafish primitive hematopoietic precursors [55]. Conversely, it does not lead to increase in proliferation of more differentiated cells in our system. The signal derived from *l-plastin*, a marker of monocytes and macrophages was not modified by NPMc+, as shown by *in situ* hybridization experiments, while anti-GFP immunostaining analysis in *mpo:GFP* zebrafish embryos injected with *NPMc+* mRNA did not show an alteration in the number of neutrophils (fig. 19).

A proliferative effect on hematopoietic progenitors was also observed after the downregulation of endogenous *npm1a*, suggesting that part of the effect of NPMc+ in the leukemogenesis processes might derive from the negative regulation of the wild-type.

### **NPMc+ has a dominant negative effect on NPM1**

Our observations provide new evidence supporting the hypothesis of a dominant negative function of NPMc+ on NPM1. We showed that the expression of NPMc+ or the knockdown of wild-type *npm1a* by injection of a morpholino gave origin to similar effects such as decreased *dkk1b* expression at the beginning of gastrulation (fig. 13) and alteration of convergence extension movements governed by non-canonical Wnt signaling (fig. 11).

Moreover, the co-injection of human *NPM1* mRNA could rescue the effect of either *NPMc+* mRNA injection or the injection of the morpholino designed against

endogenous *npm1a*. The mechanism underlying this dominant negative effect remains elusive. A possible explanation might be the delocalization of the wild-type protein from the nucleus to the cytoplasm: the injection of *NPM1* mRNA and the subsequent increase in the wild-type protein might modify protein dosage and balance, with a higher amount of wild-type protein that can remain in the nucleus and fulfill its functions.

On the other hand, the two proteins may compete for the same interactors, which, in the presence of *NPMc+*, are sequestered in cytoplasm and no longer available in the nucleus. Finally, interactions between *NPMc+* and new partners could be part of the readout of *NPMc+* presence within the cell: the different localization of *NPMc+* might help its interaction with molecules that are not physiologically bound by *NPM1*.

### ***NPM1* and *NPMc+* modulate Wnt signaling in zebrafish development**

*NPMc+* expression during zebrafish development and hematopoiesis produced effects that were in most cases rescued by the co-injection of an inhibitor of canonical Wnt signaling, suggesting that *NPMc+* might positively modulate the pathway.

*NPMc+* expression may be partially compensated by co-expression of *dkk1b* in early phases of development in determining the position of future neuroectoderm through convergence and extension movements (fig. 11). Even though *dkk1b* is an inhibitor of canonical Wnt signaling, it has been reported to be involved in convergence extension movement, which are instead governed by non canonical PCP pathway [126]. Therefore, *NPMc+* might deregulate the PCP pathway during gastrulation and its effect is partially rescued by *dkk1b* overexpression (fig. 11). Also *NPM1* overexpression has an effect on PCP pathway (fig. 11). Interestingly, the effect on the dimensions of future neuroectoderm were very similar to those observed after the

*dkk1b* injection, suggesting that NPM1 has an inhibitory effect on the pathway (fig. 11).

Activation of canonical Wnt signaling has different effects at different time points during zebrafish development. A distinction has to be made between the maternal and the zygotic Wnt pathway: in the first case, components of the Wnt signaling of maternal origin are present in the egg and the yolk and are necessary for the first phases of the zygote development. Zygotic genes are indeed expressed starting from the mid-blastula transition (MBT, around 3.5 hpf) [111], at the beginning of the maternal to zygotic transition (MZT). During MZT, zygotic products gradually replace maternal factors, which disappear within 6 hpf [142].

Canonical Wnt signaling is controlled by maternal determinants and/or zygotic factors, depending on the stage of analysis. Interestingly, Shinya et al. demonstrated that *dkk1b* is a target of pre-MBT Wnt signaling (maternal) but can negatively control the zygotic post-MBT Wnt signaling [143]. We observed a decrease of *dkk1b* expression at 30% epiboly (before MBT) (fig. 13), suggesting a negative modulation of NPMc+ on maternal Wnt signaling, which in turn might positively regulate the zygotic signaling, maybe through the decrease in *dkk1b*. Further experiments on the expression of maternal and zygotic genes have to be performed to demonstrate this hypothesis. Moreover, like NPMc+, NPM1 may modulate maternal Wnt signaling. Knockdown of the endogenous *npm1a* resulted in a phenotype similar to NPMc+ expression, characterized by a strong decrease in *dkk1b* expression, while the overexpression of the human wild-type form strongly increased and expanded the area of *dkk1b* expression (fig. 13). Therefore, NPM1 might enhance maternal Wnt signaling, still supporting the dominant negative effect of NPMc+ on NPM1.

NPMc+ and NPM1 were also able to alter zygotic Wnt pathway activation, as shown by the modulation of GFP signal in *TOP:GFP* transgenic embryos (fig. 14). In this

transgenic strain the expression of GFP is controlled by a promoter that is recognized and bound by the TCF/LEF family of  $\beta$ -catenin co-activator. Therefore, the expression of GFP is an indirect signal of canonical wnt signaling activation. In this system, NPMc<sup>+</sup> is able to increase the GFP derived signal and to partially rescue the inhibition caused by the *dkk1b* overexpression (fig. 14). *dkk1b* inhibition is rescued also by the co-injection of the morpholino against *npm1a* (fig. 14).

As shown by the partial reversion of the phenotype upon *dkk1b* co-injection, the myeloproliferative effect of NPMc<sup>+</sup> might be partially due to its ability to modulate the Wnt pathway. Whether the alteration in the hematopoietic progenitor pool is a direct effect of Wnt signaling governing hematopoiesis or a wider effect on the overall development of the embryo remains unknown: alteration of Wnt signaling might modify the cell fate at earlier stages, namely expansion of the mesendoderm region that will develop into hemogenic tissue. The importance of Wnt activation in vertebrate primitive hematopoiesis has been proposed by Tran et al: the canonical Wnt pathway, in particular the wnt4 ligand derived signal is necessary for maintenance and specification of primitive hematopoietic cells in *Xenopus laevis* [144]. Wnt signaling is, therefore, important not only in definitive and adult hematopoiesis [145] but also in primitive hematopoiesis.

### **NPMc<sup>+</sup> expression does not alter the phenotype of a murine hematopoietic stem/precursor cell line**

Despite the modulation of Wnt signaling that we observed in zebrafish development after the injection of NPMc<sup>+</sup> mRNA, we did not succeed in recapitulating these observations in an *in vitro* mammalian system.

An attempt to express NPMc<sup>+</sup> in primary murine progenitors (ckit<sup>+</sup>, sca<sup>+</sup>, lin<sup>-</sup> cells, KSL cells) was made in the lab, but the expression level of NPMc<sup>+</sup> was extremely low.

We chose to express NPMc+ in an immature cell line which can be easily differentiated toward the myeloid lineage. Moreover, NPMc+ associated leukemia is characterized by multilineage involvement: Pasqualucci et al showed that the mutation in NPM1 is present in myeloid, erythroid, and megakaryocytic cells but not in fibroblasts in NPMc+ AML patients, suggesting that the founding mutation appears in an early progenitor or a stem cell [41]. We took advantage of the EML-C1 cell line, a murine hematopoietic stem/progenitor cell line capable of differentiating along both the myeloid and lymphoid lineages.

As opposed to other hematopoietic cell lines, EML-C1 cells tolerate the expression of NPMc+ without any sign of massive apoptosis (fig. 22 and 24). However, the NPMc+ expressing cell line did not present any significant difference in growth (fig. 23), myeloid differentiation capabilities (fig. 25) or in the level of expression of Wnt genes (fig. 28) when compared to its control counterpart, the empty vector infected cell line. Moreover, EML-C1 cells appeared to be difficult to stimulate with activators and inhibitors of Wnt signaling (fig. 32-33, 36-37), leading to the conclusion that EML-C1 cells might not be the suitable model to analyze the modulation of Wnt signaling that we observed in zebrafish.

The lack of effect of NPMc+ expression may be due to the differentiation status of the cell line. EML-C1 cells are a mixture of ST-HSC (CD34+) and progenitor (CD34-) cells [146]: the effect of NPMc+ might be relevant and evident only in more undifferentiated (LT-HSC) or more differentiated (myeloid committed progenitors) cells.

Another reason may be the ratio of NPM1/NPMc+ protein dosage. As suggested in the literature, protein dosage and the balance between the wild-type and the mutant protein are important for leukemogenesis [147]. NPMc+ AML patients present a mutation in only one allele of NPMc+: a double mutation of NPM1 is not tolerated

[148]. In our *in vitro* system, NPMc+ is expressed at high levels but two copies of the wild-type version remain in the cellular genome. Moreover, the first mouse model of NPMc+ which developed leukemia was obtained by substitution of the an internal region of one of the two alleles of NPM1 with the corresponding mutated region [56], while the expression of NPMc+ in less controlled way leads to a myeloproliferative phenotype and does not result in leukemia [54]. Thus, regulation of NPMc+ expression levels needs to be finely tuned.

The lack of a physiological niche has to be considered as another possible cause for the absence of phenotype in an *in vitro* system. Chou et al. showed that perturbation of the hematopoietic niche is detected in a recent knock-in mouse model carrying a mutated murine *Npm1*. NPMc+ effect might not be cell autonomous, or only partially cell autonomous: in this case, an *in vitro* system will never be a correct method to study NPMc+ function.

Finally, the hypothesis that the ability of NPM1 and NPMc+ to regulate both the canonical and the non canonical Wnt signaling might be limited to zebrafish embryonic development, or more generally to embryonic development, should be considered. Indeed, the analyses and the study performed in the *in vivo* model are related to developing embryos, a stage in which Wnt signaling is of pivotal importance [74]. Moreover, the effect of either the presence of NPMc+ or the knockdown of *npm1a* were studied in primitive hematopoiesis and their effect could be attenuated in adult hematopoiesis.

### **Wnt signaling in NPMc+ AML blasts**

We analyzed the expression level of Wnt target genes and Wnt components in leukemic blasts from patients with NPMc+ AML. In this system, the choice of a negative control was of pivotal importance. Patients' blasts were compared to human



hematopoietic progenitors, sorted from a healthy donor on the basis on their expression of CD34, and to mature monocytes and granulocytes.

*CCND1* and *c-MYC* are indeed Wnt target genes but are very generic, and as expected, were highly expressed in CD34+ cells (fig. 41-42). On the contrary, *AXIN2* is expressed in the patients' blasts at a higher level compared to CD34+ cells (fig. 46). *AXIN2* is indeed a widely accepted target gene of the canonical Wnt pathway, forming a negative feedback loop aimed to restrict the signaling. The other two analyzed genes, *AXIN1* (fig. 47) and *CTNNB* (fig. 45), did not present the same clear trend: thus, we cannot conclude that Wnt signaling is activated by NPMc+ in blasts.

We cannot compare NPMc+ AML to other AML, given the widespread involvement of Wnt signaling in leukemias. Moreover, among the NPMc+ AML samples, the analysis is complicated by the unclear genomic landscape. NPMc+ AML is indeed associated with normal karyotype, but NPMc+ is not the only mutation found at diagnosis: mutations in *NPM1* are often associated with (*FLT3-ITD*) and mutations in *DMT3A*. We cannot, therefore, exclude that alteration of Wnt signaling and/or inhibition of the pathway might derive from other mutations acquired later during the leukemogenic process.

## **Conclusions and perspectives**

In conclusion, we describe a yet unknown function of nucleophosmin, which behaves as an inhibitor of canonical Wnt signal during vertebrate embryonic development. Its leukemogenic mutant, NPMc+, activates both canonical and non-canonical Wnt pathways and leads to an expansion of the pool of hematopoietic progenitors in a Wnt-dependent manner. Our results suggest an involvement of Wnt activation in the establishment of NPMc+ AML, as has been shown for other leukemias. In order to demonstrate that canonical Wnt signaling is involved in the

myeloproliferative effect of NPMc+, treatment of zebrafish embryos with chemical Wnt activator (LiCl, BIO) as well as inhibitors (indomethacin) will be performed. Treatment could be easily performed adding the chemical compound in the tank water as described in Yin et al. for another Wnt inhibitor [149].

Interestingly, the effect of NPMc+ and NPM1 on hematopoiesis might be an indirect effect caused by an alteration of the dorso-ventral and antero-posterior identity. In the definition of the anteroposterior identity, an important role is also played by *Hox* genes and their upstream modulators, including the caudal-related (*Cdx*) genes. Zebrafish mutants for *Cdx* genes have a bloodless phenotype, revealing their role in directing mesodermal cells toward the hematopoietic fate [150], and canonical Wnt signaling has been shown to regulate the *Cdx/Hox* axis [151]. The characteristic activation of *Hox* genes in NPMc+ AML [40] may, therefore, partly derive from activation of canonical Wnt signaling. Taking advantage of the zebrafish systems, new insights into the mechanism of alteration of *Hox* genes in NPMc+ AML will be provided.

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## APPENDIX 1

**Table 7. Patients's information.** For each NPMc+ AML patient, information regarding FAB classification and FLT3 mutation are reported.

	FAB classification	FLT3-ITD
PT1	M5a	yes
PT2	M5a	yes
PT3	M2	no
PT4	M1	yes
PT5	M4	no
PT6	M2	no
PT7	M5b	no
PT8	M2	yes
PT9	M2	no
PT10	M5b	no
PT11	M4	yes
PT12	M5a	no
PT13	M2	no
PT14	M4	no
PT15	M2	no
PT16	M5b	no
PT17	M5b	no
PT18	M1	no
PT19	M5	no
PT20	M4	no



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