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"Identification and functional analysis of genes with a role in proliferation and erythroid differentiation of a chronic myelogenous leukemia cell line"

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ABSTRACT

Identification and functional analysis of genes with a role in proliferation and erythroid differentiation of a chronic myelogenous leukemia cell line

RNA interference of gene expression (siRNA/shRNA) is a well-established technique used for loss-of-function studies and is particularly useful for identifying and validating drug discovery-relevant targets. However, for cell lines which grow in suspension (e.g. lines derived from leukemias or hematological malignancies) reliable and reproducible siRNA-mediated gene knockdown with standard liposomic reagents has to date proven difficult to achieve, due to low efficiency of oligonucleotide delivery and high sensitivity of such cells to transfection conditions.

We developed a novel approach for large-scale transfection of siRNA oligonucleotide libraries and image-based phenotypic screening which overcomes some of the limitations related to the use of non-adherent cell lines. Suitable experimental conditions were identified for efficient and nontoxic transfection of siRNA oligonucleotides in the myelogenous leukemia cell line K562. The approach was validated through screening of the K562 cells against a subset of a commercial siRNA library, targeting a subset of the druggable genome (about 6000 siRNA oligonucleotides). Results of the screen confirmed robustness of assay conditions, with hits including oligonucleotides targeting genes known to be required for K562 cell viability, such as the kinase ABL1, which is a driver

oncogene in this line, as wells as additional genes whose products are more generally required for cell viability and proliferation (e.g., PLK1 and WEE1 kinases, proteasome subunits, DNA and RNA polymerases). Among non-canonical hits, not expected a priori to inhibit cell growth, 14 genes were confirmed as novel potential molecular targets in K562, with a role in promoting proliferation/viability or, notably, in preventing terminal differentiation.

In order to further study the role of a selection of the screening hits, we established a high throughput high-content screening platform based on multiplexed imaging on a K562 cell line variant (β-K562), which expresses significant levels of β-globin in addition to the α- and γ-globins which are normally expressed in parental K-562 cells. Setup of a method for the simultaneous analysis of Hoechst DNA staining, together with immunocytochemical staining for detection of adult hemoglobin (HbA, composed of an α2βb2 globin heterotetramer) and fetal hemoglobin (HbF, composed of an α2γ2 globin heterotetamer), resulted in a robust and sensitive assay able to detect variations in γ-globin/β-globin ratio in response to drug treatments, as validated by treatment with known γ-globin inducers (hydroxyurea, butyric acid, hemin). The availability of this single cell high-content analysis assay permits investigation of the degree of population heterogeneity in response to drug treatment and to discriminate between drugs acting specifically on either γ- and β-globin expression and those acting indiscriminately on the expression of both genes.

By applying this assay to β-K562 cells transfected with siRNA oligos selected among the screening hits described above, several

genes were found to play a role in cell differentiation and hemoglobinization. Most notably amongst these, HMOX-2 (Heme oxygenase-2) silencing was shown to greatly increase γ-globin expression. We observed that also enzymatic HMOX-2 inhibition by Tin protoporphyrin-IX resulted in increased γ-globin expression, confirming Heme oxygenases as potential targets for the pharmacological γ-globin reactivation. This is of particular interest because several metalloporphyrins have already been developed for clinical uses and could be tested (alone or in combination with other HbF inducers) to improve the treatment of β-hemoglobinopathies

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Chapter1

GENERAL INTRODUCTION

Leukemic cell lines are useful models for oncology drug discovery, in particular for loss-of-function target validation studies using siRNA oligonucleotides. At present, liposome-based transfection procedures for siRNA oligonucleotide delivery in leukemic cell lines result inefficient and often toxic. Several alternative transfection methods have been developed, with pros and cons when applied to non-adherent cells (Table 1). Development of a simple and fast siRNA transfection procedure suitable for high-throughput screening would be highly desirable.

The most commonly used technique for siRNA transfection in suspension cell lines is electroporation: cell membrane permeability is temporarily increased by means of an intense electric pulse, allowing penetration of large or charged molecules into the cells. Electroporation is considered the most efficient transfection methods, but it is always associated with high cell mortality. Nucleofection is an improved electroporation method developed by Amaxa (Lonza), but it is difficult to optimize and it is not easily transferable into high-throughput settings.

Another approach uses liposomic reagents: many commercial ready-to-use liposomic mixtures have been developed and optimized for different protocols (for example, direct or reverse transfection) or even for individual cell lines. These reagents are mixtures of

positively charged and neutral lipids, like trifluoroacetylated lipopolyamine (TFA-DODAPL), dioleoyl phosphatidylethanolamine (DOPE) (Zelphati O., et al., 2001), dioleoyl trimethylammonium-propane (DOTAP) or dioleoyl sn-glycero phosphocholine (DOPC) (Simberg D, *et al.,* 2004). Although they represent useful reagents for nucleic acid delivery in cells in vitro, they often result toxic at efficacious concentrations.

Protein transduction domains are 10-35 amino acid peptides derived from HIV-TAT, HSV-VP22 or phage display libraries. These carriers contain multiple arginine and lysine residues that facilitate macromolecule transport across the cell membrane (Zelphati O., *et al.*, 2001). Unfortunately, also these reagents are not optimized for use in suspension cell lines.

Table 1 Examples of publised approaches for siRNA transfection in non-adherent cell lines

Cell lines	Transfection method	Transfection efficiency	Reference
K562/Adr	**Amaxa Nucleofector and Lipofectamine 2000 (Invitrogen)**	No significant difference of gene knockdown observed at 24 and 48hours. At greater time points, Amaxa Nucleofector induced 84.4% gene knockdown and Lipofectamine 2000 52.8%.	Lim M.N. et al. 2007
HL-60 and K562	**Nucleofection and Accell siRNA (Thermo Fisher)**	Gene expression reduction: 41-62% (Nucleofection) and 35-53% (Accell)	Østerg H. et al. 2011
Jurkat, Karpas 299 and K562	**Nanoparticles (PBAE, poly beta amino ester)**	PBAE nanocomplexes (>7kDa) with polybrene pretreatment. Plasmid delivery efficiency: Jurkat cells: 32%; K562: 19%; Karpas: 19%	Zhao N. et al. 2012
K562	**Tat-LK15 multifunctional peptide**	Tat–LK15/siRNA induced at least 70% gene silencing at 48 h post-transfection using 5-10 μg of siRNA	Arthanari, et al., 2012

Given the drawbacks of currently available methods, we aimed at the development a novel approach for siRNA transfection in

leukemic cell lines using commercial liposomic reagents. We achieved high transfection efficiency and low toxicity by transfecting cells at very low densities (100-200 cells/well) in 96-well plates using the reagent RNAiMAX®. This procedure was successfully applied for a large-scale siRNA screening of about 5800 oligonucleotides.

Drug discovery process

The drug discovery process involves a series of complex and time consuming activities aimed at the identification and development of safe and effective drugs (small molecules or biologics). This process requires huge investments, since it takes typically 10-15 years from target identification to a marketed drug (Lifeng Kang L., *et al*. 2008).

Two different strategies can be employed in drug discovery: a target-oriented approach or a target-agnostic approach. The first approach is the most diffuse and it is based on *a priori* definition of the therapeutic target, previously identified and validated. The second approach, also defined as "forward pharmacology" or "phenotypic drug discovery" strongly relies on cell-based assays, and does not envisage a particular target, but a biological effect directly in cells. Often, the primary target of drugs identified by means of this approach remain unknown. This approach is more complicated and expensive, but it has been successfully employed in the past to discovery many classical cytotoxic drugs and, recently, in drug discovery for in rare (orphan) diseases (Zheng W, *et al*., 2013).

The development of a new drug is divided in two main phases: preclinical and clinical. The preclinical phase includes i) the

identification and validation of a molecular target or cellular process involved in the pathogenetic process, ii) the high-throughput screening (biochemical or cellular) of compound libraries to identify patentable chemical classes showing target inhibition, iii) medicinal chemistry activities for rational design and improvement of compound properties, iv) confirmation of compound mechanism of action in cells and in disease-relevant animal models, v) pharmacokinetics and toxicodinamic studies. The clinical phase is further divided in four phases and it is performed to study the safety and efficacy of the experimental drug in patient populations.

Target identification and validation

A drug target is defined as any biological entity whose activity, structure or localization can be modulated by means of a small molecule or biologics. Potential targets include biomolecules and several protein families: receptors (membrane and nuclear), protein-modifying enzymes (kinases, phosphatases, proteases, ubiquitinases, deacetylases, etc.), nucleic acids, structural proteins (microtubules, actin, etc.), hormones, ion channels, and so on. The study of the molecular basis of the disease is a crucial first step in the whole drug discovery process, since poor target validation is recognized to be responsible for the failure of most drug discovery projects, even at late stages.

The aim of target identification is to find a molecular target or cellular mechanism that is causally implicated with the appearance and the progression of the disease. A typical oncology target can be, for example, a mutated or overexpressed protein that promotes uncontrolled cancer growth and whose modulation (inhibition or

activation) is believed to be beneficial for the patient. Several molecular changes may occur in pathological conditions: genetic mutations, chromosome aberrations or signaling pathways modifications (Toniatti C., *et al*. 2014).

Commonly used techniques to analyse the function of potential drug targets include analysis of molecular signaling pathways, molecular pharmacology of variants, comparative genetics, gene overexpression, antisense RNA, disease-association genetics and expression data. In the pharmaceutical industry, target identification is mainly performed by loss-of-function phenotypic screening using silencing techniques (gene knockout, RNA interference and more recently genome editing tools: Zinc finger nucleases, TALENS, CRISPR/CAS9).

RNA interference (RNAi)

RNA interference is a cellular machinery that plays an important role in sequence-specific post transcriptional gene silencing. This mechanisms can be used to study the molecular function of any gene. It is evolutionary conserved and found in plants, mammals, worms and fungi (Bhinder B, *et al.*, 2014).

RNAi can be performed by short interfering RNA (siRNA) or short hairpin RNA (shRNA). siRNA are chemically synthesized double stranded RNA (dsRNA). dsRNA oligonucleotides are introduced into the cells where they get cleaved by a RNAase III endonuclease (Dicer) into 21-23 base pair long siRNA. siRNAs are incorporated into the RNA-induced silencing complex (RISC), where the double strand is separated. Passenger strands are released into the

cytoplasm, whereas guide strands work as a template to recognize its target mRNA. The siRNA in RISC complex binds to mRNA in a sequence specific manner, resulting in the cleavage of this mRNA (Tseng YC., *et al*. 2009). Recognition of targeted mRNA is possible thanks to complementarity 3'- untranslated region (3'-UTR) of targeted mRNA with 5'-"seed region" of siRNA/miRNA. The "seed region" is localized at position 2-8 of the siRNA and it is associated with pairing specificity. RNA interference is due to degradation of mRNA, modulation of stability of targeted mRNA or inhibition of protein translation. Depending on their origin, it can be possible to distinguish different interfering RNAs: endogenous RNA, micro RNAs (miRNA), chemically synthetized siRNA or shRNA expressed by plasmids or viral vectors.

miRNAs are endogenous RNAs encoded by genomic DNA and synthesized as long hairpin transcripts which are pre-processed by the Drosha complex. miRNAs are exported from the nucleus to the cytoplasm by the exportin 5 protein, incorporated into the Dicer complex and then processed, as already described for siRNAs (Behlke MA., 2008).

While siRNA/miRNA silencing cause reversible knockdown of protein of interest, shRNA transfection allows long-term gene silencing. shRNA are exogenous RNA usually expressed by plasmids or viral vectors (mainly adenovirus or lentivirus). shRNA are transcribed into pri-miRNA (with the two strands joined by a hairpin) and processed by the Drosha complex in nucleus. miRNA are then exported from nucleus into the cytoplasm and incorporated into the Dicer complex (Moore CB., *et al*. 2010).

Once identified the target must be confirmed. The aim of target validation is to demonstrate that the target is involved in the pathological process. The ideal target should be specifically implicated in the disease of interest, not expressed or functional in normal cells and druggable (chemically "tractable") (Hughes JP., *et al.*, 2011, Vistoli G., et al., 2008). Common molecular techniques to study target expression and to confirm the cellular activity of targeting drugs by means of specific biomarkers include q-PCR (quantitative polymerase chain reaction), immunoblotting (Moore CB., *et al*., 2010), western blot and immunofluorescence.

Immunofluorescence is often exploited in multiplexed cellular assays based on image analysis, called "high-content assays". The **Array Scan V^{TI} high-content screening reader (Thermo Scientific)** is an automatic microscope able to perform quantitative single-cell analysis at the subcellular level based on imaging of fluorescent markers: it allows measurement of the intensity, localization and distribution of fluorescent signals in a cell-by-cell or field-by-field manner. This instrument is equipped with several image analysis algorithms to analyse up to 5 fluorescent signals in different cell compartments (membrane, cytoplasm, and nucleus) with one single acquisition. Common cell-based assays performed with the ArrayScan reader include cell count, DNA content analysis and nuclear shape (fragmentation, condensation) and quantification of immunofluorescently labeled markers in the nucleus or cytoplasm (Krausz E, Korn K. 2008).

SCOPE OF THE THESIS

siRNA oligonucleotide-mediated gene silencing is a common strategy exploited for target identification and validation in many therapeutic areas. Efficient silencing is generally difficult to achieve using standard liposomic reagents in non-adherent cell lines (e.g., leukemic cells) due to low transfection efficiencies and the high sensitivity of these cells to transfection reagents. Therefore, the aim of this thesis is to develop a novel technical approach for large-scale siRNA transfection in leukemic cell lines using liposomic reagents and to apply this approach to identify novel druggable targets.

The chronic myeloid leukemia cell line K562 was used as reference model. This cell line expresses the oncogenic BCL-ABL fusion protein and it is a well-characterized oncogene-driven cell line. Moreover, K562 cells are considered as representative of human erythrocyte precursors cells and have been widely used to study the molecular regulation of the expression of embryonic and fetal human globin genes. Indeed, this cell line expresses embryonic (HbE, $\alpha 2\varepsilon 2$) and fetal (HbF, $\alpha 2\gamma 2$) hemoglobin.

The **Chapter 1** describes the setup of transfection conditions and the siRNA proliferation screening. We have established suitable transfection conditions for efficient siRNA delivery in K562 cells, using commercial liposomic reagents. Then, a large-scale proliferation siRNA screening was performed using 5808 siRNA oligos targeting 1936 genes (3 siRNA oligos per gene, on average), allowing identification of 11 potential novel targets essential for K562 cell viability or proliferation. The **Chapter 2** introduces and discusses

some critical aspects of target identification/validation in the context of the oncology drug discovery process.

In **Chapter 3** we describe the characterization of a K562 clone, that we named β-K562, which expresses adult hemoglobin in addition to embryonic and fetal. We use this cell line to generate a novel high-content assay for hemoglobinization. Transfection of β-K562 cells with a set of siRNA oligonucleotides allowed identification of potential therapeutic targets whose inhibition results in increased hemoglobin expression. These targets could be exploited to develop new therapeutic opportunities to treat hemoglobinopathies (i.e., Sickle Cell Anemia and β-thalassemia).

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Chapter 2

Development of a transfection protocol for siRNA delivery in K562 cells and identification of novel targets required for K562 cell proliferation

INTRODUCTION

Chronic myeloid leukemia (CML) is a myeloproliferative disorder originating from pluripotent hematopoietic stem cells. CML represents 15% of adult leukemias (Baccarani M, *et al.*, 2013). The median age at the time of diagnosis is around 60 years, with a frequency of population 1.5 cases / 100 000 individuals.

CML is associated with high frequency to t(9,22) (q34:q11) translocation, called "Philadelphia (Ph) chromosome", which results in the expression of a constitutively active BCL-ABL fusion protein. Philadelphia chromosome is found in 95% of CML and in 15% of acute lymphocytic leukemia (ALL) patients (Shah NP, *et al.*, 2002). BCL-ABL triggers multiple signaling pathways and is crucial for growth and survival of leukemic cells. Moreover, ABL constitutive kinase activity promotes proliferation, oncogenic transformation, alters cell adhesion to stroma of bone marrow, suppresses apoptosis and promotes genetic instability.

Clinical progression of the disease is divided in three phases, according to World Health Organization (WHO): chronic phase (CP: with blast cells <15%), accelerated phase (AP, with 10-19% blasts in blood or bone marrow, or >30% blasts and promyelocytes in blood or bone marrow and >20% basophils in blood, thrombocytosis or

thrombocytopenia) and blast crisis phase (BP, >20% blast cells in blood or bone marrow with extramedullary blastic infiltration apart from spleen) (Baccarani M, *et al.*, 2013).

Standard front line therapy for Ph+ CML patients is imatinib mesylate (STI571, Gleevec), a selective, small molecule TKI (tyrosine kinase inhibitor) of c-Abl kinase, c-KIT and platelet-derived growth factor receptor (PDGFR). Imatinib binds to and stabilizes BCR-ABL kinase in its inactive conformation and, as a consequence, it impairs ATP binding and activation. Different kinds of chemical bonds are established between the drug and protein, like hydrogen bonds and Wan der Waals interactions (Gambacorti-Passerini CB, *et al.* 2003, Branford S *et al.* 2002).

Indications for imatinib treatment are: newly diagnosed cases of Ph+ CML in chronic phase of disease (400mg per day), patients with Ph+ CML in all phases of disease after interferon α failure (600 mg per day), pediatric patients with Ph+ CML in CP, newly diagnosed, after transplantation failure or resistant to interferon-α (dosage must to adapted for cases and treatment strategies should be individualized).

Efficacy of imatinib was shown in all stages of CML, causing durable cytogenetic responses. Despite the fact that in almost 50% of patients treated with imatinib in CP of disease, major cytogenetic response (MCyR) occurs, most of them are still BCR-ABL positive, as detect by polymerase chain reaction (PCR) (Sawyers C., *et al.*, 2002, Hughes TP, *et al.*, 2014). Overall survival at 5-years in imatinib treated patients is around 93%, with good drug tolerability (Baccarani M, *et al.*, 2013, Bittencourt H, *et al.*, 2008). Main side effect include:

myelosuppression, elevated liver enzymes, congestive heart failure, dysfunction, dermatologic reaction, nausea, vomiting, diarrhea, musculoskeletal pain, fatigue.

European Leukemia Net (ELN) recommended in 2013 for CML patients cytogenic and molecular test for disease progression at 3, 6, and 12 months of treatment. Good treatment response is achieved when BCR-ABL transcript level is < 10% at 3 months, <1% at 6 months and <0-1% at 12 months. Partial cytogenetic response (PCyR) should be obtained after 3 months and complete cytogenetic response (CCyR) after 6 months. Treatment with transcript levels >10% at 6 months and >1% at 12 months define treatment failure, as well as lack of PCyR from the 3rd months and CCyR at month 6 (Baccarani M, *et al.*, 2013). Despite good response to imatinib treatment, more than 45% of patients fail therapy after 8 years. Most of patients at BP of disease still respond to imatinib treatment, but nearly 70% of them relapse within 3-6 months. Only 25% of patients in AP respond, but relapse often occurs (Sawyers C., *et al.*, 2002). It has been observed that BCL-ABL imatinib resistance mutations occur in around 30% of patients (17% of primary, 13% of secondary), which eventually determine disease progression (Breccia M, *et al.*, 2008).

Primary imatinib resistance in Ph+ patients (called "refractoriness") is associated with lack or very weak clinical response to the treatment. Two main cellular mechanisms for TKIs resistance have been identified: BCR-ABL gene amplification and point mutations. BCR-ABL gene amplification causes increased kinase protein levels and can be overcome by dose escalation or treatment with second generation TKIs. In case of primary BCL-ABL-

mutated clones already existing prior to treatment initiation, the therapeutic regimen is more difficult. Igbal, *et al.* performed experiments on CD34+ progenitor/stem cells derived from 100 newly diagnosed CML patients in CP, identifying 32 patients with mutations in the kinase domain (KD). The most frequent were: F311L (50%), M351T (87.5%) and T351I (37.5%) median follow-up to relapse in this group was around 30 months (range between 8 to 48 months) (Iqbal Z, *et al.*, 2013).

Secondary resistance to imatinib (also referred to as ”acquired resistance”) may occur in patients that previously obtained complete cytological response. In these cases, TKI resistance could be caused by BCR-ABL gene amplification or mutations in different domains of the protein. Gene amplification is associated with kinase overexpression in around 10% of relapsed patients (Shah NP, *et al.*, 2002, Gambacorti-Passerini CB, *et al.* 2003). However, the main causes of imatinib resistances are point mutations that are present in around 90% of relapsed patients. More than 100 point mutations were identified in BCR-ABL; most of them in the kinase domain (Iqbal Z, *et al.*, 2013, Khorashad JS, *et al.*, 2008), localized close to the ATP binding pocket. Moreover, in some patients after failure of imatinib treatment it has been observed co-existence of polymutated and unmated cells (Gibbons DL, *et al.*, 2014, Shah NP, *et al.*, 2007). This finding suggests clonal selection and cytogenic evolution in leukemic cells: BCR-ABL mutated cells can arise independently and preexist before diagnosis (Shah NP, *et al.*, 2002, Gambacorti-Passerini CB, *et al.* 2003, Gibbons DL, *et al.*, 2014).

Shah and al (Shah NP, *et al.*, 2002) sequenced kinase domain of 45 CML imatinib resistant patients: 90% of mutations were found in KD. The most frequent (60% cases) were: E255K, T315I and M351T. A second group of mutations were localized in the p-loop (ATP- phosphate binding loop) G250E, Q252H, Y253F/H, and E255K that are involved in conformational changes responsible of imatinib displacement. A third group was shown to occur within the activation loop: E355G, M351T, V379I. Different mutations may have heterogeneous effects on imatinib resistance. Amino acid substitutions in position T315I and E255K are associated to complete imatinib resistance G250E is associated to high resistance, Q252H, F317L, M351T and E255G to moderate resistance. Patients bearing the third group of mutations could still benefit from imatinib treatment at higher doses.

Imatinib is unable to completely eradicate mutated blast cells; therefore the only opportunity to cure CML is allogenic stem cell transplantation (alloSCT) or bone marrow transplantation (BMT). Unfortunately, SCT is correlated to high mortality, mainly as a cause of graft versus host disease (Baccarani M, *et al.*, 2012, Deininger M, *et al.*, 2006). Retrospective data shows that 5-years overall survival in newly diagnosed patients in CP of disease is higher in imatinib-treated patients (93%), than in patients subjected to SCT (59%, P<0,001). Moreover, data have shown no influence of imatinib treatment on SCT (Bittencourt H, *et al.*, 2008, Deininger M, *et al.*, 2006). For this reason, as long as patients respond to tyrosine kinase inhibitors, the best therapeutic option is continuation of pharmacological approach. AlloSTC could be considered in young patients (under 40), as it

results in highest overall survival, decreased morbidity and mortality. ELN recommends transplantation (both alloSCT and BMT) only in patients who do not respond to TKIs, or carrying the T315I BCL-ABL mutation (Baccarani M, et al., 2013).

Nilotinib, dasatinib, bosutinib are the current approved second line tyrosine kinase inhibitors for Ph+ CML patients, after resistance or prior intolerance to imatinib treatment, in all phases of the disease.

Nilotinib (Tasigna) is an imatinib analog targeting BCR-ABL, c-KIT, PDGFR, Src-family kinases (SFKs). Tasigna is an orally bioavailable drug which shows higher potency and selectivity against BCR-ABL than imatinib, also in the case of some imatinib-resistant mutations. The drug was approved in 2007 for the treatment of Ph+ CML patients in chronic and accelerated phase of disease, resistant or intolerant to imatinib. Phase 3 of clinical, randomized, multicenter study on 846 patients showed faster and more pronounced response to nilotinib treatment than imatinib. After 12 months of treatment, nilotinib (300 mg twice a day), demonstrated major molecular response (MMR) in 44% of patients, the double as compared to imatinib (22%, 100 mg daily). CCyR in nilotinib patients was obtained faster than with imatinib after 12 months, moreover, the response was more prolonged (Saglio G, *et al.*, 2010). Adverse effects include: rash, headache, purities, alopecia, elevation of liver enzymes (AST, ALT, total bilirubin level), as well as grade 3 and 4 thrombocytopenia, neutropenia, and anemia.

Dasatinib (Sprycel) is multikinases inhibitor targeting BCL-ABL, Src family kinases, PDGFR, c-KIT and ephrin kinase receptor (EPHs). Dasatinib demonstrates higher *in vitro* antiproliferative

activity than imatinib and nilotinib, also in the presence of some imatinib-resistant BCR-ABL mutations. Nevertheless, cells carrying T315I or E255 BCR-ABL mutations are resistant to dasatinib (Hanaizi Z, *et al.*, 2014), whereas V299L, T315A, and F317L/V/I/C mutations are sensitive only to elevated dose of dasatinib. The drug binds to the active form of the enzyme, for this reasons it is able to overcome most of imatinib resistant mutations (Shah NP , *et al.*, 2007). Randomized clinincal phase 2 trials in newly diagnosed Ph+ CML patients showed that dasatinib is more efficient than imatinib. Sprycel induced faster cytogenic and molecular responses from 6 month onwards. Despite of the faster and more prolonged responses observed after dasatinib treatment, there was no influence on overall survival. The toxicological profile of this TKI is more severe that imatinib, but on the other hand, it was demonstrated that dasatinib and nilotinib target a broader spectrum of ABL kinase mutations.

Bosutinib (Bosulif, SKI-606) was approved by FDA as a second line treatment in CML patients, in chronic, accelerated or blast crisis phase in which imatinib, dasatinib and nilotinib are not recommended. Bosutinib is a Src/ABL tyrosine kinase inhibitor showing also modest inhibitory activity against c-KIT kinase. The drug shows activity in all phases of CML, except in the presence of some BCR-ABL mutations including: L248R, G250R, E255K/V, V299L and T315I. Side effects include: 1/2 grade gastrointestinal events, diarrhea, hepatotoxicity, rash, cardiac arrhythmia with QT prolongation and fluid retention. Bosulif received approval after having demonstrated efficiency in II clinical phase, where a group of 52 patients resistant to dasatinib (carrying mutation in F317 or E255)

and nilotinib (E255, Y253, F359) demonstrated MCyR in 18/36 patients in chronic phase and objective responses in 7/16 patients in advanced phase of disease (Hanaizi Z, *et al.*, 2014). A German multicenter study confirmed these data on a group of 118 patients resistant on dasatinib, nilotinib and imatinib, in CP. A follow up at 28.5 months in 32% of patients showed MCyR, 24% CCyR, and 73% CHR (Khoury HJ, *et al.*, 2012).

Ponatinib is pan-TKI active against CML cells and Ph+ ALL, active also against the T315I BCR-ABL mutation, that was approved as second line therapy in imatinib resistant patients, and was applied as a third line of therapy (Baccarani M, *et al.*, 2013, Cortes JE, *et al.*, 2013). Ponatinib received initial approval after phase 2 clinical trial in December 2013. The trial was performed on 449 patients (412 with CML, in all 3 phases of disease), who were resistant or badly tolerated treatment with second generation TKIs (dasatinib, nilotinib) or developed mutation M315I after TKI treatment. 56% of patients in CP, 39% in AP and 23% BP-CML showed MCyR, that in 91% remained for at least 12 months. Moreover, no BCL-ABL mutation demonstrated resistance on ponatinib. Unfortunately, treatment with ponatinib induces multiple side effects. The most frequent hematological adverse events are: thrombocytopenia (37%), neutropenia (19%), and anemia (13%). Non hematological side effects include pancreatitis (69%) that tends to occur at the beginning of treatment but it is reversible. Life-threatening site effects include: cardiovascular, cerebrovascular and peripheral vascular events as a consequence of thrombotic and blood clots (Cortes JE, *et al.*, 2013). Nowadays ponatinib sale is suspended of adverse events.

MATERIALS AND METHODS

Chemicals and transfection reagents

Lipofectamine RNAiMAX Transfection Reagent, OptiMEM® I Reduced-Serum Medium and DEPC-treated water (pyrogen free) were obtained from Invitrogen, (Life Technologies, Carlsbad, CA, USA), Foetal Bovine Serum (FBS) was purchased from Euro Clone (Italy). Transfection reagents were bought from different manufacturers: RiboCellin BCC- Bio Cell Challenge, (SAS, France), Hylimax (Dojindo Laboratories, Kumamoto, Japan), SAFEctin™ Deliverics (Edinburgh, Scotland, UK) and Lullaby OZ Biosciences (SAS, Marseille, France). Phosphate Buffered Saline (PBS), RPMI 1640 medium and PenStrep antibiotics (10 000 Units/mL Penicillin, 10 000µg/mL Streptomycin) were purchased from Gibco® (Life Technologies™, Thermo Fisher Scientific Inc, Carlsbad, CA, USA) Propidium Iodide, Hoechst 33342, formaldehyde hydroalcoholic Solution, (37% wt. in water), NaCl, from Sigma- Aldrich (St. Louis, MO, USA).

The human Silencer Select Druggable Genome siRNA Library V4 was obtained from Ambion Life Technologies (Carlsbad, CA, USA). A non-targeting siRNA oligonucleotide (NTO), and two oligonucleotides respectively targeting the 26S proteosome subunit ATPase 3 (PSMC3), and the kinesin family member 11, (KIF11/Eg5 were synthesized in house. siRNA oligos contain a 2′-O-methyl ribosyl substitution at position 2 of the sense strand to significantly reduce off-target effect (Watts JK and Corey DR, 2012). Their sequences are reported in Table 2.

Table 2 siRNA oligos sequences

siRNA oligo	Sense stand sequence
NTO	5'-UGGUUUACAUGUCGACUAAtt-3'
PSMC3	5'-CGGCUGAAGUGCGCAAUAA-3'
KIF11/Eg5	5'-CUGAAGACCUGAAGACAAUdTdT-3'

Cell cultures and treatments

The human chronic myeloid leukemia cell line K562 obtained from the European Collection of Cell Cultures (ECACC) was grown in RPMI 1640 medium supplemented with 10% of heat-inactivated FBS (v/v), 1% (v/v) Pen-Strep antibiotics at a density of 500 000 cells/ml. Cells were kept humidified atmosphere with 5 % CO_2 at 37˚C, passages were performed twice a week.

Preparation of lipid formulations and transfections

DOPE (1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamione), DOPC (1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine [C44H84NO8P]) and DOTAP (,2-dioleoyl-3-trimethylammonium-propane (chloride salt) [C42H80NO4Cl]) liposomes were obtained from Lipoid GMBH (Ludwigshafen, Germany).

Lipids were weighted and dissolved in $ChCl_3$ to obtain the stock solution in concentration 10 mg/ml. 2 mg of lipids were evaporated to dryness using a vacuum pump, at room temperature. 700 µl of Hepes buffer 2X (composed by 306 nM NaCl and 40 mM of Hepes) were added and whole mixture was sonicated for 5 minutes. A same volume of water was added to obtain standard osmolarity and sonicated for further 5 minutes. Lipids were mixed in different proportions to obtain a final concentration of 3 mg/ml.

5 µl of lipids were mixed with 80 µl of Hepes buffer 1X, 10 µl of control siRNA oligos 120 nM (NTO or PSMC3) and incubated for 10 min. After incubation 200 µl of OptiMEM was added, mixed gently and incubated for another 5 min, then 900 µl of cell suspension (80000 cells/well) were added and incubated at 37°C for 72h.

Flow cytometry

Cells were analyzed by flow cytometry for DNA content by propidium iodide (PI) staining. Samples were washed twice in PBS, stained with 50 µg/ml of PI in PBS supplemented with 12 µg / ml RNase and 0.02% Nonidet-P40. Cells were incubated for 30 min in the dark. The experiment was performed in triplicate using Modfit® 3.0 software (Verity Software House).

siRNA transfection setup

A series of experiments was conducted to find optimal (nontoxic and efficient) experimental conditions for siRNA reverse transfecion of K562 cells in U-bottom 96-well plates

Cells were transfected using different transfection reagents (HilyMax, SAFEctin, Ribocellin, Lullaby and RNAiMAX) following manufacturer's recommendations. Briefly, siRNA oligonucleotides were resuspended in serum-free medium OptiMEM® at a concentration of 20 nM prior incubated for 5 minutes at room temperature, then lipid transfection reagents prepared at increasing concentrations in OptiMEM® medium (0.5, 1, 1.5, 2, 3, 6 and 10 µl/ml) were added, mixed gently and further incubated for about 20 minutes.

100 µl of cells at a density of 1500 ml were added to transfection mixtures (final density 150 cells/well). After 7 days of incubation, cells

were fixed for 20 minutes with 3.7 % formaldehyde, stained with Hoechst 33342 (1:500) and transferred to flat-bottomed 96-well CELLSTAR® Black / μClear® plates. Plates were spun for 5' at 2g to left cells attach to plate bottom, then were analyzed with Array Scan reader (Thermo Fisher Scientific) to count the cells per field as proliferation parameter.

Transfection efficiency

Optimal conditions were estimated using control siRNA oligonucleotides: a negative control (NTO) and two positive controls, which are expected to induce cell death (PSMC3 and Eg5), all synthesized in house. Experimental conditions were considered optimal when the positive siRNA controls induce > 70% of cell death (high efficiency) and the negative control NTO induces < 25% cell death (low toxictity).

Immunofluorescence and high-content analysis

The Array Scan VTI high-content screening reader (Thermo Fisher Scientific) is an automated, inverted epifluorescence microscope (Zeiss, Thornwood, NY) equipped with image analysis software. This high content screening platform allows automated, quantitative cellular and subcellular analysis, with high sensitivity. Image acquisition is supported by autofocus and autoexposure hardware.

For proliferation assays cells seeded in 96 well plates were fixed, stained with the DNA dye Hoechst and acquired in the Channel 1 (Ch1, blue fluorescence) (365WB50 filter). The image analysis algorithm was set to recognize individual nuclei based on Hoechst staining and to calculate for each one the area and shape, the mean

fluorescence intensity and the spatial coordinates within the field. Cell debris, artifacts or cell clusters were excluded from the readout. All images were stored in a dedicated server for the subsequent analysis. Cell number per well was assessed by calculating the average cell count per field in at least 10 fields (parameter "Selected Cell Count Per Valid Field"). Any other calculation was performed using Microsoft Excell.

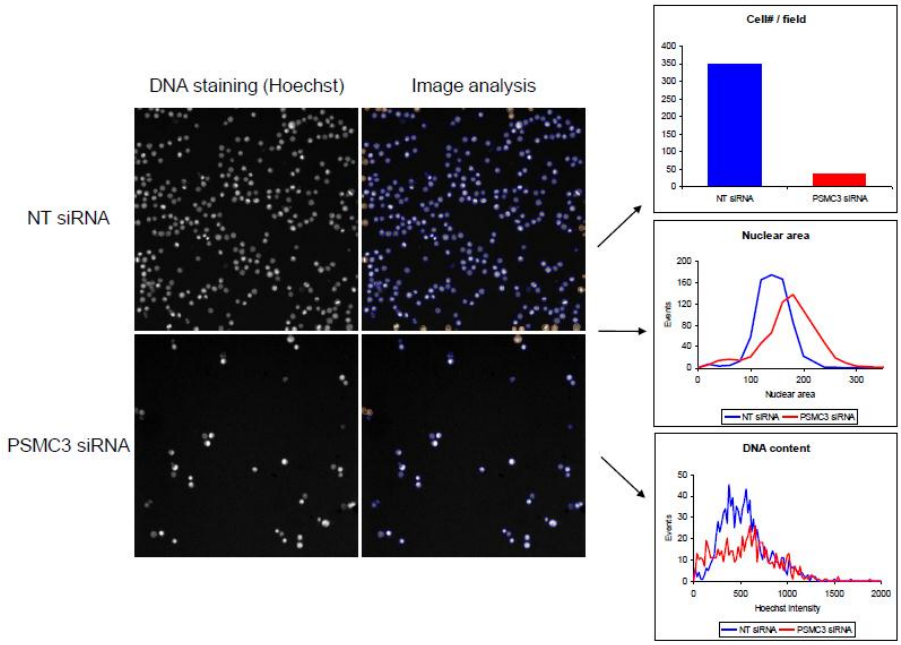


Figure 1 High content analysis of siRNA phenotype, representative photos
Representative fields of K562 cells transfected with the indicated siRNA oligonucleotides and stained with Hoechst 33342 (left) and graphical representation of three parameters obtained from image analysis (right): cell count per field, nuclear area distribution and DNA content distribution.

K562 proliferation screening of the siRNA oligonucleotide library

K562 cells were transfected with a subset of human Silencer Select Druggable Genome siRNA Library V4 (Ambion Life Technologies). Sixty six 96-well plates containing unique lyophilized siRNA oligos (0.25 nmol/well), targeting 1932 genes (5796 siRNAs, three oligos per gene with different sequences). All oligos were dissolved with 100 µl of DEPC-treated water (pyrogen free) to prepare stock solutions 2.5 µM. Eight empty wells of twelfth column in every plate were filled with control siRNAs: NTO and PSMC3 (3 wells for each control). Controls were added manually, at the concentration of 2.5 µM. Stock plates were further dissolved with DEPC-water in U-bottom 96-wells plates to obtain 100 µl/well of oligo solution at a concentration of 800 nM (mother plates). For each plate, 17 µl of oligos were transferred into second U-bottom plate (daughter plate) and filled with 119 µl of OptiMEM® I medium containing 4 µl/ml of Lipofectamine RNAiMAX. The solution was mixed gently, incubated for 20 minutes and 30 µl/well were transferred into a U-bottom plates. After incubation 60 µl of cell suspension, containing 1667 cells/ml (100 cell/well) were added to transfection mixture (final siRNA oligo concentration: 20 nM). All liquid handling and plate barcode tracking were performed using Freedom EVO® robotic platform (Tecan, Männedorf, Switzerland). Plates were incubated for 7 days at 37°C in humidified atmosphere of 5% CO_2. After incubation, cells were fixed with 3.7 % formaldehyde for 20 minutes, stained with Hoechst 33342 (1:500) and transferred to black, flat-bottomed 96-well plates, centrifuged and counted with the ArrayScan VTI platform applying the Nuclear Translocation algorithm to count the cells per well.

Screening data analysis

The cell count per field parameter by ArrayScan analysis was normalized as % with respect to the average of three values of NTO in every plate. No further normalization, trimming or data corrections were applied. Microsoft Excel (Redmond, WA, USA) was used for statistical analysis and visualization of screening data distributions. Statistical analysis included also Z-score factor (median ± a MAD method).

RESULTS AND DISCUSSION

1. Optimization of siRNA transfection conditions for K562 cells

With the aim of identifying optimal conditions to transfect the leukemic cell line K562, notoriously difficult to transfect in high-density microplates with standard liposomic reagents, we explored several experimental variables, including: different types of liposomic transfection reagents (commercial or prepared in house), different transfection reagent concentrations, different siRNA oligonucleotide concentrations, different cell densities and plate effect.

1.1. Effect of different commercially available reagents on transfection efficiency

The toxic effect of different commercially available lipids for transfection was estimated. The highest cell viability and response linearity were obtained after Lipofectamine RNAiMAX transfection. Then diverse mixtures for transfection, composed by different proportions of commercially available lipids, were performed. Lipofectamine RNAiMAX showed the highest transfection efficiency, calculated as a difference between NTO and PSMC3, as compared in house prepared transfection lipids (Fig 2A) To establish optimal RNAiMAX concentration inducing the highest transfection efficiency and minimal toxicity in K562 cells, we performed dose- response experiments. Cells were transfected by serial dilutions of Lipofectamine RNAiMAX at the following concentrations: 1, 2, 4, 8 and 10 μl/ml. The experiment revealed that the highest transfection efficiency achieved between 4-8 μl/ml of RNAiMAX, for

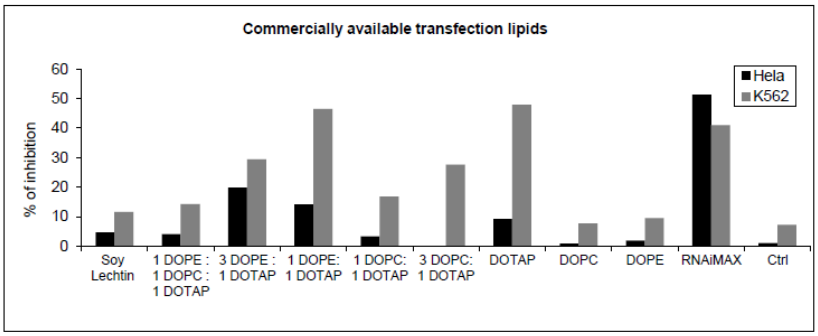
proliferation assay (Fig 2B). It is demonstrated that high concentration of siRNA oligos increase the risk of, targeting different genes in addition to the primary targeted causing off-target effects and introducing false positive (Watts JK and Corey DR, 2012). To reduce this possibility, cells were transfected with siRNA concentrations in the range of 10 - 20 nM (Fig 2C).

Cell growth is characteristic for every single cell line and depends on duplication time. Cell density is a crucial factor to optimize for siRNA transfections, therefore we performed experiments at different cell densities: 100, 150, 200, 250, 300 and 500 cells/well. Optimal conditions were achieved at 100–200 cells/well (Fig 2D).

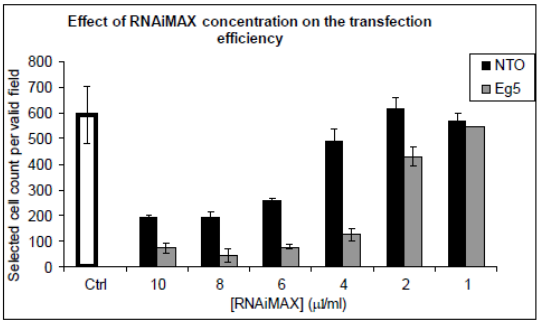
We performed transfection experiments in K562 in 96-well plates with different geometries: flat bottom (Greiner Bio-one, Germany) and U-bottom (Greiner Bio-one, USA). Interestingly, our data showed higher transfection efficiency in U-bottom plates (Fig 2E).

The optimal siRNA transfection conditions identified by set up experiments are the following: Lipofectamine RNAiMAX transfection reagent at a concentration of 4-6 μl/ml in the initial OptiMEM® solution;10-20 nM final siRNA oligo concentration; cell density in the range 100-200 per well and incubation time of 7-10 days. The whole process is performed in U-bottom plates, then cells are transferred to flat bottom plates for analysis.

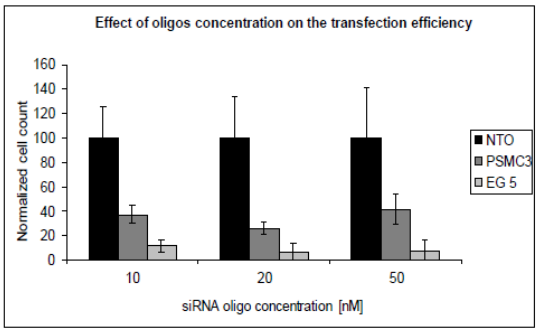
A


Commercially available transfection lipids
% of inhibition
60
50
40
30
20
10
0
Hela
K562
Soy Lechtin
1 DOPE : 1 DOPC : 1 DOTAP
3 DOPE : 1 DOTAP
1 DOPE: 1 DOTAP
1 DOPC: 1 DOTAP
3 DOPC: 1 DOTAP
DOTAP
DOPC
DOPE
RNAiMAX
Ctrl

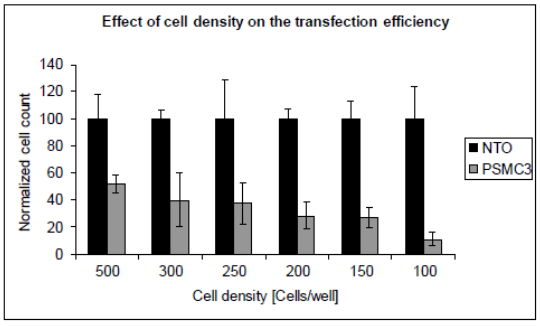
B


Effect of RNAiMAX concentration on the transfection efficiency
Selected cell count per valid field
800
700
600
500
400
300
200
100
0
NTO
Eg5
Ctrl
10
8
6
4
2
1
[RNAiMAX] (µl/ml)

C


Effect of oligos concentration on the transfection efficiency
Normalized cell count
160
140
120
100
80
60
40
20
0
NTO
PSMC3
EG 5
10
20
50
siRNA oligo concentration [nM]

D


Effect of cell density on the transfection efficiency
Normalized cell count
140
120
100
80
60
40
20
0
NTO
PSMC3
500
300
250
200
150
100
Cell density [Cells/well]

E

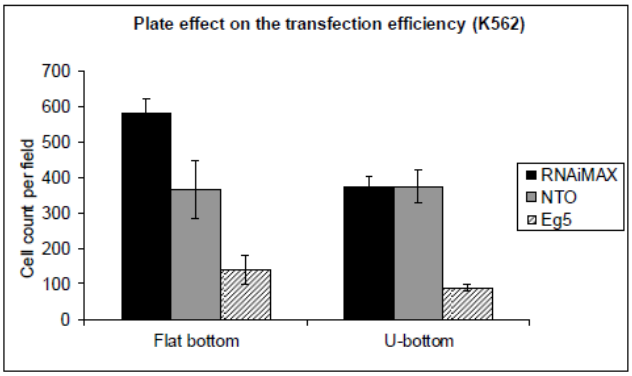


Figure 2 Optimization of siRNA transfection conditions for K562.
The effects of several experimental variables on transfection efficiency and toxicity of three reference standard siRNA oligonucleotides (NTO, PSMC3 and Eg5) were evaluated in K562 cells. All transfections were performed using a "reverse" protocol in 96-well plates and 7 days incubation time (see text for details). A) K562 cells and HeLa cells, for comparison, were transfected in parallel with PSMC3 siRNA oligo 20 nM using different liposomic transfection reagents at the same concentration Which; the % cell growth inhibition (normalised on NTO) is reported. B) K562 cells were transfected with NTO and Eg5 siRNA oligos 20 nM in the presence of increasing concentrations of RNAiMAX transfection reagent. The cell count was analyzed with the ArrayScan reader (average ± standard deviation of three experiments). C) K562 cells were transfected with different siRNA oligonucleotides concentrations in the presence of 0.1% RNAiMAX (cell count was normalized on NTO samples; average ± standard deviation of three experiments). D) K562 cells were transfected at increasing densities (from 100 to 500 cells/well) with NTO and PSMC3 siRNA oligos in the presence of 0.1% RNAiMAX. E) Effect of plate geometry on transfection efficiency: K562 cells were transfected with the indicated siRNA oligonucleotides or only in the presence of RNAiMAX reagent in flat bottom or U-bottom plates.

1.2. Pilot screening

The optimal transfection conditions identified were transferred to a robotic liquid handling station (Freedom EVO®, Tecan, Männedorf, Switzerland) for large-scale automated transfection and siRNA screening. Pilot screening was performed to validate the experimental conditions and to test the robotic workflow, including plates and reagents positions on the workbench or the time needed to transfect a single plate. To confirm robustness of the whole process,

we transfected K562 cell with four plates containing 352 siRNA oligos (one siRNA oligo per gene), including oligos targeting some genes known to be required for K562 proliferation like: ABL1 (kinase rearranged in the BCR-ABL oncogene which is expressed and driver in this cell line) and PLK1 (Polo-like kinase 1, a mitotic kinase) (Radich JP, *et al*. 2006, Bhinder B., 2013). All data were normalized, within any plate, to the average values of three NTO oligos.

Pilot screening results revealed highly efficient siRNA delivery into the cells, based on the antiproliferative activity of positive controls (PSMC3 siRNA oligo). Transfection efficiency was 69 % ± 12.32 %, calculated as a difference between normalized, average values of NTO and PSMC3. The efficient cell killing induced by ABL1 and PLK1, in addition to PSMC3 silencing, in view of modest toxicity of the Non Targeting Oligo (28%, as compared to non-transfected cells) demonstrated that the transfection protocol established for K562 is suitable for large scale screening (Fig 3).

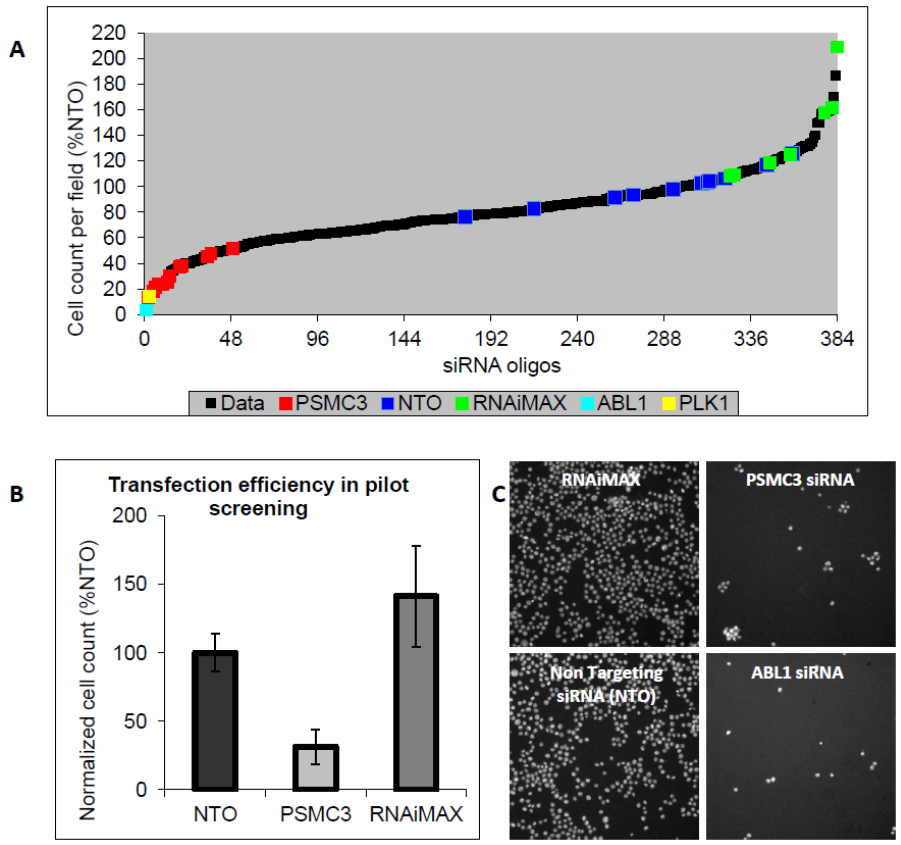


Figure 3 Pilot screening results reveal highly efficient siRNA delivery
A) Pilot screening proliferation results in K562 cell line: the cell counts per field (normalized on NTO) are shown for individual oligos. Relevant oligonucleotides are highlighted in color. B) Results of control samples: NTO, PSMC3 and RNAiMAX (average ± standard deviation of three experiments). C) Representative ArrayScan fields of K562 cells transfected with the indicated oligonucleotides and stained with Hoechst 33342.

2.1. siRNA proliferation screening

A proliferation screening was conducted on the chronic myeloid leukemia cell line K562, searching for genes which are essential for proliferation or viability of this cell line. We screened the antiproliferative activity of 5808 siRNA oligos targeting 1936 genes (3 siRNA oligos per gene, on average). Cells were transfected in U-bottom 96-well plates and incubated for 7 days, then fixed and stained

with Hoechst 33342 (DNA dye). After transfer to flat-bottom plates, the cells were analyzed with the ArrayScan high-content screening reader to count the cells per field in all wells as a proliferation-related parameter. Proliferation data were normalized on the basis of the average value of three NTO samples (set as 100%) in all plates. Transfection efficiency was estimated for every single plate, as well as for the whole plate set. The average NTO value resulted 100% (Standard Deviation = 29.4), whereas PSMC3 siRNA 8.91% (Standard Deviation = 6.0). This standardization procedure allows data comparison between different plates.

A threshold was arbitrary set at 20%NTO to define hits: any siRNA oligonucleotide inducing >80% cell loss was considered as a hit in our proliferation assay. The frequency distribution of proliferation results of the 5808 siRNA oligos and the controls (NTO and PSMC3) is shown in Fig 4.

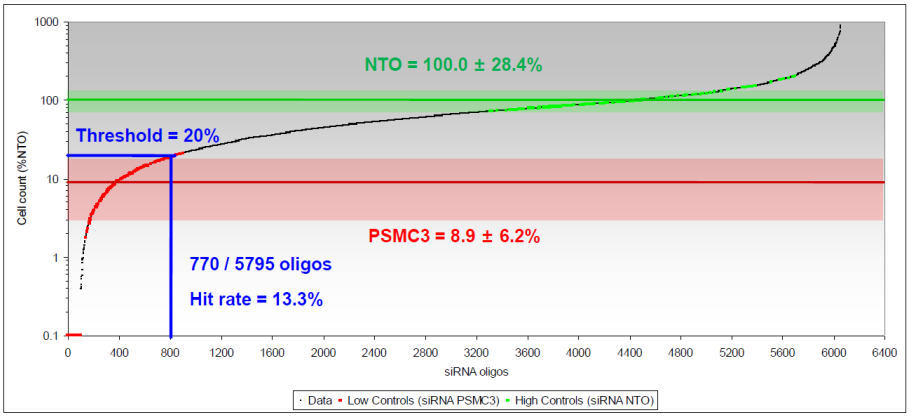


Figure 4 Proliferation screening results
Negative control samples (NTO) are evidenced in green, whereas positive controls (PSMC3) in red. A blue line represents the threshold applied to define hits.

Our screening allowed identification of 37 genes for which three oligos out of three showed >80% K562 cell number decrease as compared to NTO and 91 genes with two oligos out of three were active. Genes with only one oligo out of three showing antiproliferative activity were not considered as potential targets and were excluded from further analysis. Among the hits we found proteins and enzymes regulating fundamental cellular processes, like: proteosome subunits (23 hits out of 29 screened), DNA and RNA polymerases, small nuclear ribonucleoproteins and kinases like (ABL1, GAK, HCK or PLK1). Most of them have been already described in literature as genes required for proliferation of K562 cell line (Bhinder B., 2013, Luo J, *et al.*, 2009).

The genes emerged with 3/3 active oligos are highly significant “top” hits. They include, among others, the kinases ABL1, HCK, GAK, several proteasome subunits, DNA and RNA polymerases and some members of the spliceosome complex.

The BCR-ABL1 oncoprotein is the main driver of K562 cell line and promotes cell growth, proliferation, survival and migration. Silencing of Abelson kinase 1 (ABL1), but not ABL2, resulted in K562 apoptotic cell death.

HCK (Hematopoietic Cell Kinase) is a member of the Src tyrosine kinases family, that is expressed exclusively in primary hematopoietic cells, particularly in myeloid and B-lymphoid lineages. The BCR-ABL kinase directly interacts with three members of Src tyrosine kinase family: HCK (Warmuth M, et al., 1997), LYN and FGR (Danhauser-Riedl S., et al., 1996). This interaction has been demonstrated in cell lines, where Src kinases are implicated in

proliferation and cell transformation. In patients overexpression of HCK or LYN is associated with disease progression (Hoshino K, *et al.*, 2007). However, our siRNA screening identified HCK, but not LYN and FGR, among top hits.

The cyclin G-associated kinase (GAK, auxilin 2), is a regulator of clathrin-coated vesicle trafficking. This protein mediates clathrin binding to the plasma membrane. Knock down of Gak is associated with cell cycle arrest in metaphase due to impaired spindle assembly. Moreover, Gak is responsible for centrosome integrity and chromosome congression, and has also a role in endocytosis (Shimizu H., *et al.*, 2009).

The proteasome is responsible for the degradation of short-lived, destroyed or incorrectly spiced proteins involved in many different cellular processes, like cell cycle control, DNA repair or apoptosis. Moreover, the proteosome is also involved in DNA repair and unknown, non-proteolytic functions during transcription (Reed SH, *et al.*, 2007). Proteasomes are composed by a catalytic 20S core and two regulatory 19S subunits. The core is a highly conserved, barrel-shaped structure, formed by 4 heteromeric rings, arranged in a sequence alfa-beta-beta-alfa. Each ring is composed by 7 subunits, called PSMA 1-7 and PSMB 1-7. PSMCs and PSMDs subunits are regulatory particles which form the two lids of the barrel. The proteosome core is always composed by the above-mentioned subunits, except immune cells, where subunits PSMB1, PSMB2 and PSMB5 are respectively replaced by PSMB8 (PSMB5i), PSMB9 (PSMB1i) and PSMB10 (PSMB2i) (Luo B, *et al.*, 2008). The results of our screening show that the silencing of canonical proteosome

subunits (PSMAs, PSMBs, PSMCs and PSMDs) but not of the immunoproteasome subunits (PSMB8, PSMB9 and PSMB10), results in K562 proliferation inhibition, as expected.

DNA polymerases are a group of 15 enzymes involved in DNA synthesis, replication, repair and genetic recombination. Their function is crucial for all cell lines. The DNA polymerases PolA, PolD, PolE are directly involved in DNA replication. PolA is composed by 4 subunits: PolA1 (catalytic), PolA2 (regulatory), Prim1 and Prim2 (respectively: small and large subunits). PolA initiates DNA synthesis by providing RNA primers, the polymerases PolD and PolE are responsible for their prolongation. They have a role also in different DNA repair pathways, (nucleotide excision repair, mismatch repair, double strand breaks repair). The DNA synthesis process is very efficient and the possibility of mismatch mutation in case of these enzymes in estimated to be less than 10^{-7} - 10^{-9} per base pair (Heitzer E, Tomlinson I. 2014, Lange SS, *et al.*, 2011). Polymerase sub-family members also include PolB (responsible for gap filling and DNA synthesis during base excision repair), PolG (mtDNA-associated polymerase), PolH (it has important functions during replication since it promotes bypass of damaged DNA (Lange SS, *et al.*, 2011). siRNA oligonucleotides targeting ten DNA polymerases were present in the library screened. Screening results revealed that silencing of PolA1, PolD, PolE1 and PolE2 is lethal for K562 cells. Moreover, significant antiproliferative effects were also observed following PolD1, PolG and Prim2 silencing, which, as a consequence of the stringent threshold that we applied, were not considered as hits.

DNA-dependent RNA polymerases are a family of enzymes present in all living organisms which use DNA as a template for RNA transcription. For this reason, RNA polymerases are crucial for cell homeostasis, growth, differentiation, and development. Three classes of RNA polymerases have been identified: Pol-I class members transcribe ribosomal RNA (rRNA); Pol-II members transcribe mRNA and snRNA; Pol-III family members transcribe transfer RNA (tRNA), 5S rRNA and small RNAs. All polymerases are required for cell survival, and indeed we identified four of them among screening hits with 3/3 active oligos.

The spliceosome is a multiprotein complex responsible for removing non coding sequences, introns, from pre-mRNA transcripts and join exons in a process called splicing. The spliceosome is composed by snRNA (small nuclear RNA) and several proteins. The core is composed by seven proteins: B (B' – is the alternatively splicing), D1, D2, D3, E, F, G which are assembled with snRNA. All spliceosome subunits are involved in post-transcriptional mRNA modifications (Chari A, *et al.* 2008). siRNA-mediated silencing of most of spliceosome subunits present in the library screened. Nine out of eleven screened genes resulted in K562 cell death, except silencing of SNRPA and SNRPN.

A total of 128 genes resulted hits of our screening: 45 of them (4 DNA polymerases, 10 RNA polymerases, 9 small nuclear ribonucleoproteins and 22 proteosome subunits) were not further confirmed because we considered them as “expected hits”, based on literature data or on their fundamental role in cellular processes. Rather, their presence among screening hits represents an important

cross-validation of screening results and confirms that the experimental conditions employed allow identification of genes essential for K562 cell proliferation.

WEE1, USP39 (Ubiquitin specific peptidase 39 responsible for mRNA splicing) and CDK11B (WEE1 homolog, cell cycle regulator of G2 to M transition), are genes frequently found among the top scoring hits of proliferation si/shRNA screens published in literature (Bhinder B., Djaballah H., 2013)

2.2.. Screening hits confirmation

Thirty-two siRNA oligos resulting hits of our proliferation screening were prioritized for technical confirmation. The target genes do not include members of the protein families mentioned in the previous chapter, that we consider "expected" as they frequently result top scores of published siRNA proliferation screens in different cell lines. For technical confirmation of screening results, we performed manual transfections in K562 cells using the same experimental conditions and siRNA oligonucleotide sequences of the screening. Similarly, results of confirmation experiments were normalized with respect to NTO values obtained from the same plates. Our results revealed a good correlation between screening and confirmation data: we calculated a linear correlation coefficient R^2 of 0.95 for 29 out of 32 siRNA oligonucleotides, whereas 2 out of 32 oligos were not confirmed, corresponding to a confirmation rate of 93.7%. 11 confirmed genes emerged with 3/3 oligos were confirmed as required for K562 proliferation or viability, shown in the Fig 5:

IKBKB, LONP1, NPSR1, NUP98, PITRM1, PNN, QARS, RAN, RANGAP1, USP39 and WEE1.

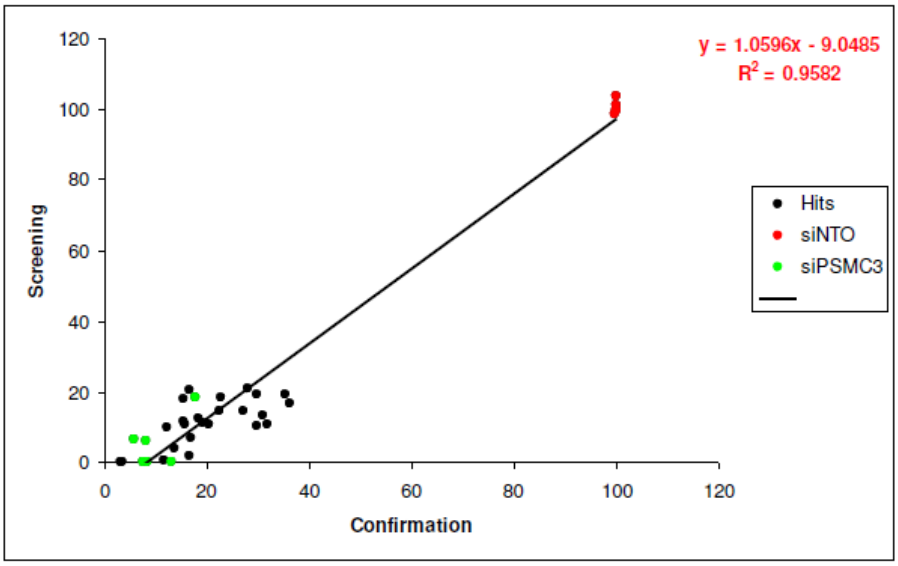


Figure 5 Technical confirmation of 29 siRNA oligonucleotides targeting genes essential for K562 cell proliferation.
The proliferation results of confirmation transfection were plotted against the results from the screening for the respective siRNA oligo. Negative control samples (NTO) are evidenced in green, whereas positive controls (PSMC3) in red

RAN is a G protein member of the Ras oncogene family involved in active transport of RNA and proteins from nucleus to the cytoplasm, through the nuclear pore complex. Its function is associated to DNA synthesis, cell cycle progression and mitotic spindle formation. RANGAP1 (Ran GTPase activating protein 1) plays a role in nuclear-cytoplasm transport machinery and, like RAN, also in mitotic spindle formation. RANGAP1 promotes Ran GTPase activity and both constitute key regulators of the nuclear export machinery.. In the cytoplasm, RANGAP1 hydrolyzes the GTP bound to RAN, converting Ran in a GDP-bound state. The opposite process occurs in the nucleus and it is promoted by the guanine nucleotide

exchange factor RCC1 (Regulator of Chromosome Condensation 1) which converts Ran in a GTP-bound state. This exchange is crucial for active transport between the nucleus and the cytoplasm. We confirmed that siRNA-mediated RAN silencing results in K562 cell death: interestingly, it has been shown that RAN silencing acts as an imatinib sensitizer and impairs colony formation assay of CD34+ cells of newly diagnosed CML patients (Khorashad JS, *et al*., 2015). RANGAP1 is highly expressed in different hematological malignancies: B-lymphoblastic lymphoma/leukemia, Burkitt lymphoma, Hodgkin's lymphoma.

NUP98 (Nucleoporin 98) is a subunit of the nuclear pore complex. NUP98 is part of Nup98-RanBP3-Xpo1-RanGTP nuclear signal cargo protein complex. To note, structural chromosomal rearrangements in this gene are well documented in hematological diseases like AML, MDS, ALL, bilineage / biphenotypic leukemia and CML in blast crisis (Gough SM, et al., 2011). It is noteworthy that both Nup98, Ran and RANGAP1 are confirmed hits of our siRNA screening, strongly implicating the nuclear export machinery as an essential process to support K562 cell proliferation. Ran emerged among the hits of a shRNA screening performed in K562 by Khorashad et al (Khorashad JS, *et al*., 2015).

IKBKB (Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-Cells) gene encodes for a serine kinase which phosphorylates the inhibitor in the inhibitor/NF-kappa-B complex, resulting in the activation of NF-kappa-B. Deregulation of this pathway has been associated to many inflammatory diseases and cancers. It has been already shown that inhibition of the NFKB pathway in leukemic cells

like (AML, ALL, CML or MDS) induces apoptosis. It is known that BCR-ABL expression constitutively activates NFKB through IKBKB: our results indicate that IKBKB function is essential in a Philadelphia chromosome-positive cell line K562 (Cilloni D., *et al.*, 2006, Lounnas N, *et al.* 2009).

Two mitochondrial proteins were identified among the confirmed hits of our screening: LONP1 (Lon peptidase 1) and PITRM1 (pitrilysin metallopeptidase 1).

LONP1 is a mitochondrial, ATP-dependent matrix protein responsible for selective degradation of misfolded or oxidative destroyed proteins, as well as for regulation of mitochondrial gene expression. LONP1 has been identified as an essential gene for cell survival and proliferation in lung fibroblasts (Bota DA, et al., 2005). The role of this gene is not yet fully understood, but it is frequently found overexpressed in cervical cancer cell lines and patients. Interestingly, LONP1 silencing in HeLa cells inhibits proliferation (Nie X, *et al*., 2013, Bayot A, et al., 2014).

PITRM1 is an ATP-dependent protease involved in proteins degradation in mitochondria; its exact function still remains unknown.

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Chapter 3

Development of high-content screening assay for the identification of modulators of globin level

INTRODUCTION

Hemoglobinopathies

Sickle cell disease (SCD) is autosomal recessively inherited hemoglobinopathy. SCD occurs in malarial endemic regions, mainly in African origin populations, and less commonly in the Mediterranean, Latino, East Indian and Arab descent.

SCD is genetically associated with point mutations in the β-chain gene for example the Val6Glu substitution. As a consequence of missense mutations, anomalous globin S (HbS, α2 β s2) is expressed in place of the normal, adult hemoglobin (HbB, α2 β2).

Clinical symptoms of SCD include vaso-occlusive episodes with multiorgan ischemia injury and inflammation. Chronic hemolysis is associated with high concentrations of unbounded hemoglobin and free iron in blood. Moreover, nitric oxide (NO) released from erythrocytes interferes with vascular homeostasis. Among the complications associated with vaso-occlusive episodes are: acute chest syndrome, central nervous system disease, renal failure, vascular necrosis, leg ulcers and frequent infections (mainly in child).

Homozygous (HbSβ0) or heterozygous (HbSS) patients manifest the most severe course of disease with very low level of Hb and frequent vaso-occlusive crisis. Hemoglobin S is poorly soluble and polymerizes

when deoxygenated. Dynamic oxygen exchange in erythrocytes causes membrane remodeling, demonstrated as a change in shape, rigidity and shorter lifespan of cells. Fetal hemoglobin interferes with polymerized of hemoglobin S (Ballas S, et al. 2012, Fucharoen S., et al., 2013).

The hemoglobin in the healthy population is composed by α and non-α components. One of the therapeutic strategies to treat SCD is to equilibrate the imbalance between α/ non- α globin chains, for example through reactivation of HbF production. Increased synthesis of γ globin can compensate imbalance in β-like chains, decreasing globin polymerization and precipitation. Clinical observations confirm that increase in HbF can ameliorate SCD syndromes (Ballas S, et al. 2012, Fucharoen S., et al., 2013, Uda M., et al., 2008).

Thalassemia consists of a group of autosomal recessive diseases, caused by point mutation in β globin gene on chromosome 11. β−globin chain synthesis in this patients can be reduced (β+) or absent (β0). The disease is prevalent in Mediterranean countries, Middle East, Central Asia, India, Africa and South of China. Among European Countries, thalassemia mainly occurs in Cyprus (14% of the whole population) and Sardinia (10.3%). It has been estimated that about 1.5% of the global population carries β-thalassemia mutation, with about 60,000 symptomatic individuals born annually.

Three main forms of disease are distinguished: thalassemia major, intermedia and minor. Patients vary from severe, mild, up to an asymptomatic course of disease, depending on genetic status (homo- or heterozygous).

Thalassemia major is symptomatic within the first two years after birth and requires regular red blood cell transfusion, as a consequence of anemia. Hemoglobin level is usually under 6 g/dl (norm: 12-16 g/dl for women and 14-18 g/dl for men). Untreated patients have growth retardation, pallor, poor musculature, skeletal changes (expansion of bone marrow), hepatosplenomegaly, jaundice, leg ulcers, dilated myocardiopathy, liver fibrosis and cirrhosis, chronic hepatitis and dysfunction in endocrynological function.

Thalassemia intermedia is usually later diagnosed, anemia and regular red blood cell transfusion is required occasionally. The hemoglobin level in these patients is in the range of 6-9 g/dl. The disease is manifested by growth retardation, anemia, and rarely: extramedullary erythropoiesis, bone deformations, osteoporosis, erythropoietic masses affecting spleen, liver and lymph nodes, increased predisposition to thrombosis (Ballas S, et al. 2012, Attie KM, et al., 2014).

Thalassemia minor is usually asymptomatic, with moderate anemia and hemoglobin level above 9 g/dl.

More than 200 point mutation have been identified in thalassemia (Fucharoen S., et al., 2013), associated with vary hemoglobin anomalies:

- Mild HbE/β-thalassemia, observed in 15% of all cases in Southeast Asia, with Hb levels in the range 9- 12 g/dl. Those patients are asymptomatic and do not require treatment.
- Moderately severe HbE/β-thalassemia, with Hb levels at 6-7 g/dl and the clinical symptoms are similar to thalassemia intermedia. Transfusions are not required unless Hb level decrease.

- Severe HbE/β-thalassemia: with very low Hb level at around 4-5 g/dl. Patients in this group manifest symptoms similar to thalassemia major.

Patients with HbC/β-thalassemia may live free of symptoms and be diagnosed during routine tests, based on the anemia and enlargement of the spleen. Blood transfusions are not required. Microcytosis and hypochromia are found in every case. The blood smear shows distinctive Hb C crystals with straight parallel edges, target cells, and irregularly contracted cells with features of thalassemia such as microcytosis.

The only way to treat sickle cell disease and β- thalassemia is bone marrow transplantation (Fucharoen S., et al., 2013, Walters MC, et al., 2000). Unfortunately, transplantation is associated with high risk of rejection, as well as with graft-versus-host disease (GvHD). This approach is available in developed countries, even where accessible HLA-matched donors are limited (Walters MC, et al., 2000, Negre O, et al., 2015).

Other treatment options in thalassemia patients include:

1) Blood transfusion

Transfusions are necessary, usually from the second year after birth, in thalassemia major patients and occasionally in adult patients with thalassemia intermedia. Transfusion is applied to ameliorate anemia, inhibit extramedullary erythropoiesis and reduce intestinal iron absorption. Blood should be administrated every three weeks in symptomatic patients. Constant medical supervision and regular transfusions permits survive up to 40 years in β-thalassemia major patients. Insufficient medical care and lack of transfusions result with

premature death, usually as a consequence of cardiac complications. Imbalance in α/β chains causes hemochromes and stimulates apoptosis of erythroid precursors. Total erythrocytes production is diminished and overall cell survival decreased. Increased iron absorption from intestine, together with regular blood transfusion results with iron overload. A consequence of total saturation of transferrin leads to unspecific iron binding that accumulates in tissues, resulting particularly toxic to the endocrine system.

2) Splenectomy

Removal of the spleen is performed mainly in thalassemia major patients, which often develop splenomegaly as a consequence of numerous transfusions, iron overload and ineffective erythropoiesis. Recent data reports that patients who overcome splenectomy encounter serious complications associated with pulmonary hypertension, heart failure, thrombosis differently from thalassemia intermedia patients not subjected to this surgery (Ballas S, et al. 2012).

3) Modulation of γ-globin level

Induction of HbF synthesis can reduce the disproportion in α / non- α globin chain imbalance ratio and mitigate the severity of disease, by improvement in erythropoiesis. Increased γ globin level reduces morbidity and mortality (Ballas S, et al. 2012, Fucharoen S., et al., 2013, Uda M., et al., 2008).

At present, hydroxyurea (HU, hydroxycarbamide) is the only drug approved by FDA to decrease severity of the crises in β- thalassemia and SCD adult patients. Hydroxyurea reduces but not abolishes vaso-occlusive crisis and chest syndromes. The exact mechanism of action of HU is still unknown, although its role as stressor agent for

erythropoiesis has been considered (Ronchi A. and Ottolenghi S. 2013). HU mechanism of action probably also includes NO generation, inhibition of in guanylyl cyclase and cyclic guanosine monophosphate depended protein kinase pathways. A double-blind randomized clinical trial, in patients with SCA (HbS β0 and HbS β+ patients were excluded from trial) showed decreased crisis frequency in patients treated with hydroxyurea in comparison with placebo, with prolonged time to the occurrence of the first crisis. HU treatment showed additional advantages: less chest syndrome episodes, less transfusions performed, increase in mean corpuscular volume (MCV), fetal hemoglobin level (HbF), and percentage of F-cells. The treatment did not cause any important adverse effects, during the trial of 21 months (Charache S, et al., 1995). Unfortunately, long time duration trial could promote myelodysplastic syndromes, leukemia (mainly polycythemia vera) and other hematological complications.

Another potential agent considered in the 80's as a potential drug was 5-azacytidne, which is DNA-methylation inhibitor. Few clinical trials were conducted with this drug, showing the following dose-limiting adverse events of this drug like: cytotoxicity, mutagenicity, immuno- and myelosuppression, activation of latent viruses (DeSimone J., et al., 1982).

Short fatty acids like butyric acid induce the expression of γ-globin synthesis with reduced level of free hemoglobin in the serum (Atweh GF, et al., 1999). Long term clinical trials with butyric acid could not be completed because of its marked antiproliferative effect on the bone marrow, despite general good tolerability (Atweh GF, et al., 1999). The observed bone marrow toxicity compromised

approval for thalassemia. Another short fatty acid derivative HQK-1001 (Sodium 2,2 dimethylbutyrate) is currently in clinical phase I/II. In a study performed on 21 thalassemia patients (14 HbE/ β0 and 7 β+/β0), for 8 months in 4 doses (10, 20, 30 and 40 mg/kg/day) HQK-1001 induced an increase of total hemoglobin levels, induction of HbF, and appearance of F-cells. The side effects weren't different from placebo (Fucharoen S., et al., 2013).

4) Potential molecular targets for targeted therapy of thalassemia

Several transcription factors have been implicated as regulators of the expression of globin genes, or γ / β-globin gene switch. Some of them are currently considered as potential targets for targeted therapeutic intervention in thalassemic patients: KLF1, BCL11A, SOX6 and COUP-TFII.

KLF1 (Erythroid Kruppel-like factor 1) is a zinc finger transcription factor required for β-globin synthesis. KLF1 gene expression levels are found 3-fold increase in differentiated erythroid cells, as compared to erythroid progenitors, in a mouse model. Moreover, chromatin immunoprecipitation assay (ChIP) showed that KLF1 increased expression is associated to its binding to the promoter of beta globin, that is a crucial step in hemoglobin switching from embryonic/fetal to adult (Tallack MR., et al., 2010, Charache S, et al., 1995).

Knockdown of this transcription factor results in increased γ- globin gene expression in human erythroid progenitor cells. KLF1 works bidirectional. It directly activates β globin expression through binding to its promoter (that is associated with indirect repression of γ-globin), and it indirectly promote expression of BCL11A by binding

to BCL11A promoter in bone marrow erythroid cells (Borg J., et al., 2010).

The BCL11A gene encodes for a Kruppel multi-zinc finger transcription factor that acts as a transcriptional repressor and is expressed in erythroid precursors. The exact mechanism of action of BCL11A is still unknown.

Genome wide association studies revealed that BCL11A variants influence on γ- globin levels in the SCA and β-thalassemia patients, as well as in healthy population. Moreover, the genotype of the patients with high levels of HbF is associated with low expression of BCL11A (Uda M., et al., 2008). Further study on BCL11A showed that the full length of BCL11A is expressed in bone marrow of adults, whereas shorter variants of this protein are expressed iprenatally (in fetal erythroblast during the second trimester and the first trimester of circulating erythroblasts). siRNA knock-down of BCL11A results in increased level of HbF mRNA in human erythroid progenitor cells, whereas shRNA knock-down in these cells shows strong increase in γ-globin synthesis (Xu J, et al., 2010). BCL11A expression is depended to KLF1 activity. Knock down of KLF1 results in decreased expression of BCL11A at mRNA and protein level (Borg J., et al., 2010). Moreover, BCL11A regulates γ- globin synthesis in collaboration with SOX6, through binding to the LCR (Locus Control Region). Probably, this protein-protein interaction may be involved in chromatin looping creation (Xu J, et al., 2010). BCL11A collaborates also with COUP-TFII transcription factor.

SOX6 belongs to the SRY family of HMG box transcription factors. It was suggest that SOX6 acts as a repressor of γ- globin genes

expression. Overexpression of SOX6 results in cell differentiation, maturation and hemoglobinization, associated with a decreased proliferation. SOX6 binds to the human ε- and γ- globin gene promoters, in K562 cell line, as shown by chromatin immunoprecipitation (ChIP) assay. The same experiment performed in cord blood-derived CD34+ cells revealed absence of SOX6 in immature progenitors, whereas during cell differentiation SOX6 start to be detected. Overexpression of Sox6 in these cells results in faster differentiation (to reticulocytes) and β- globin increase (Cantu' C., et al., 2011).

COUP-TFII is tan orphan nuclear transcription factor that belongs to the steroid / thyroid hormone receptor super family. COUP-TFII binds to γ-globin promoters, whereby it acts as a repressor of γ-globin gene expression. Knock-down of Coup-TFII in primary human erythroblasts induces γ globin expression (Aerbajinai W., et al., 2009). The exact mechanism of action and involvement in globin regulation is still unknown.

Other transcription factors involved in γ / β- globin gene switch are: GATA-1, cMYB, SOCS3, Ikaros, FOP, miR15, miR16, MBD2, NF-E4, NRF2, Tr2/Tr4.

5) Gene therapy

The gene therapy approach is based on the construction of a viral vector that in safe and controlled manner is able to efficiently integrate into the human genome and provide the wild type copy of a mutated gene, with therapeutic effect. Different vectors have been proposed to induce globin synthesis: lentiviral vectors containing the β- globin gene, or the γ-globin gene, or vectors containing γ- globin

genes with shRNA against γ- globin repressors like BCL11A or KLF1 (Cao A., et al., 2011).

Philippe Leboulch and co-workers successfully developed a gene therapy approach using lentivirus vectors containing β-globin genes with its introns and LCR (the commercial name of this therapy is LentiGlobinTM) (Negre O, et al., 2015).

6) Other drugs of potential impact on hemoglobinopathies treatment

Recently a new drug for anemia treatment reached clinical phase II: sotatercept (ACE-011) is a first-in-class biological drug consisting in a fusion protein of the extracellular domain of activin receptor type IIA (ActRIIA) conjugated with the Fc of human IgG1. In clinical studies sotatercept was shown to increase red blood cells, hematocrit and total Hb level in erythrocytes, in a dose-dependent manner (from 0.1 mg/kg once on 3 weeks) and decreased total bilirubin level. The drug is being developed as therapeutic for anemia and β-thalassemia in transfusion-depended and in transfusion-independent patients. Despite of a good drug tolerability, ACE-011 was discontinued because of adverse events or lack of response in 6 out of 25 patients (Sherman et al., 2013). The drug is currently in clinical phase II for anemia in patients with low -or intermediate-1 Risk MyeloDysplastic Syndromes (MDS) or non-proliferative Chronic MyeloMonocytic Leukemia (CMML).

ACE-536 is another drug that is currently in clinical phase I. It is a modified immuno-conjugated activin receptor type IIB (ActRIIB) used to treat erythropoietic disorders, including β-thalassemia. The drug was tested on 32 post-menopausal women, at 2 doses (0.0625

and 0.25 mg/kg) by subcutaneous injection once in 2 weeks. Non severe adverse events were observed (Attie KM, et al., 2014).

The mechanism of action of both ACE-011 and ACE-536 is to promote late-stage erythroid differentiation through binding to TGF-β ligands and inhibition of Smad 2/3 transcription factor signaling pathway (Attie KM, et al., 2014).

MATERIALS AND METHODS

Chemicals and antibodies:

Hydroxyurea, Hemin, butyric acid, Triton X-100, Butyric Acid, Hoechst 33342 and formaldehyde (37% w/v, hydroalcoholic solution) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Blotting-grade blocker (dry milk) was purchased from Bio-Rad Laboratories, USA. Imatinib mesylate (Cat. No. S1026), dasatinib (Cat. No. S1021) and doxorubicin (Cat. No. S1208), entinostat (MS-275) and dacinostat (LAQ-824) were obtained from Selleck Chemicals (Houston, TX, USA). Phosphate-buffered saline (PBS), fetal bovine serum (FBS), RPMI 1640, Opti-MEM® I medium and PenStrep antibiotics solution (10000 Units/mL Penicillin, 10000 μg/mL Streptomycin) were purchased from Gibco Life Technologies (Carlsbad, CA, USA). Hemoglobin γ antibody (51-7) PE (sc-21756 PE) and hemoglobin β antibody (37-8) FITC (sc-21757 FITC), Tin Protoporphyrin IX dichloride (sc-203452) were obtained from Santa Cruz Biotechnology (Dallas, TX, USA). APC-conjugated anti-CD235 (glycophorin) antibody was purchased from BioLegend (San Diego, CA, USA). FITC-conjugated mouse IgG_1 (Cat. No. 555748) and PE-conjugated mouse IgG_1 (Cat. No. 551436) antibodies (clone MOPC-21) were purchased from BD Biosciences Pharmingen (San Jose, CA, USA). Hemin was dissolved in 1M KOH, pH was adjusted to 7.5 with Tris-HCl solution, and then boiled for 5 minutes.

Cell cultures and treatments

K562 cell line (obtained from ECACC) and its subclone β-K562 expressing γ and β hemoglobin (obtained from Prof. G. Ferrari, San Raffaele Hospital, Milano) were grown in RPMI 1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat inactivated FBS (v/v), 2 mM L-glutamine and 1% PenStrep solution (v/v) in humidified 5% CO_2 atmosphere at 37°C.

Growth curves were obtained by seeding in duplicate K562 and β-K562 cells at a density of 5-10 × 10^3 cells/ml in 12-well plates. Cells were counted every 24 hours by a Coulter Counter (Backman Coulter) and doubling times were calculated in exponential phase of growth. Clonal origin of β-K562 cells from K562 cell line were confirmed by short tandem repeat (STR) fingerprinting by using the AmpFISTR Identifiler Plus PCR Amplification kit (Applied Biosystems) as already described (Somaschini A, *et al.*, 2013).

For chemical treatment experiments 5 x 10^4 cells were exposed to increasing doses in 24-well plates by manual serial dilution. Cells were incubated for 4 days at 37°C and then analyzed by RT-PCR or high content analysis. An experiment was done with at least three biological replicates per experiment.

Light microscopy

K562 and β-K562 cells were stained with May- Grunwald-Giemsa methods (Brown AB, 1993) and images were acquired by using an Axioplan 2 microscope (Zeiss, Thornwood, NY). Images were processed by using AxioVision software V4.8.2.0 (Zeiss).

RNA isolation and RT-PCR

Total RNA from 10^6 cells was extracted with the TRI Reagent, treated with RQ1 DNase for 30 min at 37°C and retrotranscribed by High Capacity cDNA Reverse Transcription Kit. Negative control, without reverse transcriptase, gave no signal. Real time analysis was performed using the ABI Prism 7500, (PE Applied Biosystems). Primers were designed to amplify 100 to 150 bp amplicons on the basis of sequences from the Ensembl database (http://www.ensembl.org/). Samples from two independent experiments were analyzed in triplicate. Specific PCR product accumulation was monitored by SYBR Green dye fluorescence in 25-µl reaction volume. Dissociation curves confirmed the homogeneity of PCR products. The primers used are listed in Table 3

Table 3 List of primers used for RTqPCR

Primers	Fwd sequence	Rev sequence
GAPDH	5'ACGGATTTGGTCGTATTGGG	5'TGATTTTGGAGGGATCTCGC
α globin	5'GAGGCCCTGGAGAGGATGTTCC	5'ACAGCGCGTTGGGCATGTCGTC
β globin	5'TACATTTGCTTCTGACACAAC	5'ACAGATCCCCAAAGGAC
γ globin	5'CTTCAAGCTCCTGGGAAATGT	5'GCAGAATAAAGCCTACCTTGAAAG
ε globin	5'GCCTGTGGAGCAAGATGAAT	5'GCGGGCTTGAGGTTGT

Flow cytometry

Cells were harvested, counted, washed in PBS and fixed in 3.7% formaldehyde for 20 minutes at room temperature. 10^6 cells were washed and permeabilized in 0.1% Triton X-100 in PBS for 10

minutes on ice, then washed and incubated in staining buffer (PBS + 1% milk) for 20 minutes. Cells were pelleted and resuspended in 100 µl of staining buffer containing anti-β-globin-FITC, anti-γ-globin-PE (or the FITC and PE corresponding isotypes IgG1 controls and incubated overnight at 4°C. Cells were washed in PBS and analyzed with a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

siRNA transfections

β-K562 cells were transfected as previously described for parental K562 (paragraph 1.1, page 36).

Immunofluorescence and high-content analysis

Preliminary experiments were performed to optimize the immunofluorescence protocol for the detection of β- and γ-globin in fluorescence microscopy or flow cytometry. In particular, cells were fixed for 20 minutes in formaldehyde (final concentration 3.7%), then permeabilized, and stained using different conditions: 1h incubation at 37°C in 1% BSA and 1% milk, 1h staining at 4°C in 1% BSA and 1% milk, overnight staining at 4°C in 1% w/v BSA and overnight staining at 4°C in 1% w/v milk. The best staining conditions were achieved by incubating the primary antibodies for approximately 16 hours (overnight) at 4°C in 1% w/v milk blocking buffer.

After chemicals treatment or siRNA oligonucleotides transfection, cells were fixed with 3.7% formaldehyde for 20 minutes, washed with PBS and permeabilized for 30 minutes with a PBS solution containing 0.05% v/v Triton® X-100 and 1% w/v powdered

milk (staining buffer). After a second wash with PBS, cells were incubated overnight at 4°C with staining buffer containing anti-γ, anti-β hemoglobin antibody and Hoechst 33342. PE or FITC-conjugated IgG_1 isotypes were used as negative controls. Cells were washed and resuspended in PBS, and then 100 μl/well were transferred to 96-well plates. Plates were spun for 5' at 2g to facilitate cell attachment to the well bottom, sealed and analyzed with the Array Scan VTI high-content screening reader (Thermo Fisher Scientific). At least 600 cells were acquired in each well with a 20X objective in three fluorescence channels (blue, green and red). The Molecular Translocation Bioapplication was used to determine the cell count per field, the nuclear area and intensity (based on Hoechst staining in the blue channel) and the cytoplasmatic fluorescence intensity of b (green) and g (red) globins.

Laser scanning microscopy

For multiplexed β+γ globins and Glycophorin A staining, cells previously incubated with β and γ antibodies, were further incubated for 2 hours with an APC-conjugated anti-CD235 antibody diluted 1:100 in PBS, then washed with PBS. Cells were transferred to a microscope glass slide, mounted with Mowiol (Sigma-Aldrich) and acquired with a laser scanning confocal microscope LSM710 (Zeiss).

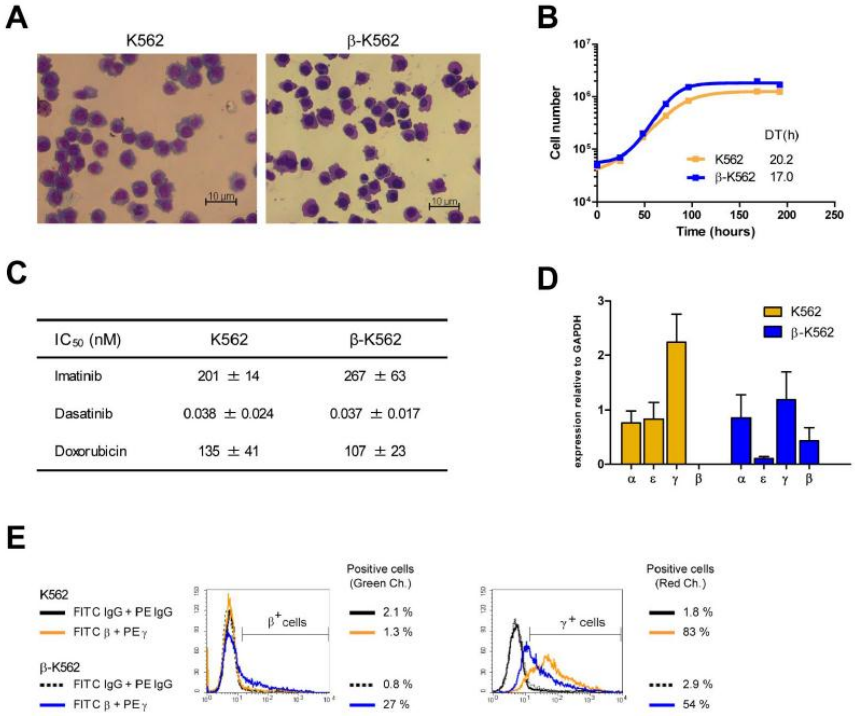
RESULTS AND DISCUSSION

1. Identification and characterization of a variant K562 subclone expressing β-globin

K562 cell line, established by Lozzio and Lozzio from a patient with chronic myelogenous leukemia (CML) in blast crisis (Lozzio CB, *et al.,* 1975) is commonly used as a model of human "erythroid" cell. In addition to its broad application in the oncological drug discovery, as a cell line model for BCR-ABL oncogenic rearrangement, K562 have been also widely used to study the molecular regulation of the expression of embryonic and fetal human globin genes (Guerrasio A, *et al.,* 1981, Rutherford TR, *et al.,* 1979). A main limitation of this cell line in hemoglobinization studies is its "fetal-like" hemoglobin pattern: K562 exclusively expresses embryonic (HbE, $\alpha 2\varepsilon 2$) and fetal (HbF, $\alpha 2\gamma 2$) hemoglobin.

While characterizing different K562 subclones with respect to their expression of globin genes, we came across a variant clone expressing the adult β-globin gene, that we named β-K562. Comparative analysis of β-K562 and K562 ECACC demonstrated morphological similarity by May-Grunwald-Giemsa **s**taining (Fig 6A), similar growth curve profile (Fig 6B) and similar sensitivity to known BCR-ABL tyrosine kinase inhibitors (imatinib mesylate and dasatinib) as well as to cytotoxic agent doxorubicin (Fig 6C). Moreover, β-K562 were characterized by DNA fingerprinting (short tandem repeats) comparison with K562 profile gave a CIS ("CLIFF Identity Score, (Somaschini A, *et al.,* 2013) of 89.2% meaning that β-K562 is substantially, corresponding to a K562-derived clone. Despite their

bona fide "K562-like" profile, β-K562 do express the adult β-globin chain, as assessed by both qRT-PCR and FACS analysis (Fig 6D), with β expression accounting for about 20% of β-like globin expression. Given its unique globin expression profile, β-K562 cell line was employed to set up an immunofluorescence based high content assay with the aim of searching new genes/drugs modulating hemoglobinization and, in particular, the γ to β ratio.



IC_{50} (nM)	K562	β-K562
Imatinib	201 ± 14	267 ± 63
Dasatinib	0.038 ± 0.024	0.037 ± 0.017
Doxorubicin	135 ± 41	107 ± 23

Figure 6 Characterization of the the β-K562 subclone by comparison with ECAAC-K562

A) May-Grunwald-Giemsa staining. B) Growth curves (n=2). C) Response (IC50) to imatinib mesylate, dasatinib and doxorubicin (n≥3). D) RTqPCR on α, ε, γ and β globins. Histograms show the relative levels of expression normalised on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and (n≥4). E) FCM analysis: cells were stained with anti γ- and anti β-globin antibodies and with the corresponding isotype controls and read in FL-1 (FITC, green channel) or in FL-2 (PE, red channel). A representative experiment is shown

2. 1. **Immunofluorescence set up for high content analysis of β and γ globins**

To perform high content analysis of globin expression, we analyzed different staining conditions. β-K562 were fixed with formaldehyde, then stained with anti-γ, anti-β or anti-γ+anti-β– globin antibodies and Hoechst 33342 in buffer containing 1% BSA or 1% of milk for 1h at 37 degrees or overnight at 4 degrees. The highest cytoplasmatic and the lowest background signals were obtained after an overnight (approximately 16 hours) staining in buffer containing 1% w/v milk (Fig 7). This procedure was used to prepare samples for further analysis with the Array Scan VTI high-content screening reader (Thermo Fisher Scientific), where different image analysis algorithms and different exposure times were compared.

The Molecular Translocation Bioapplication revealed to be the most robust image analysis algorithm, minimally affected by changes in the exposure time.

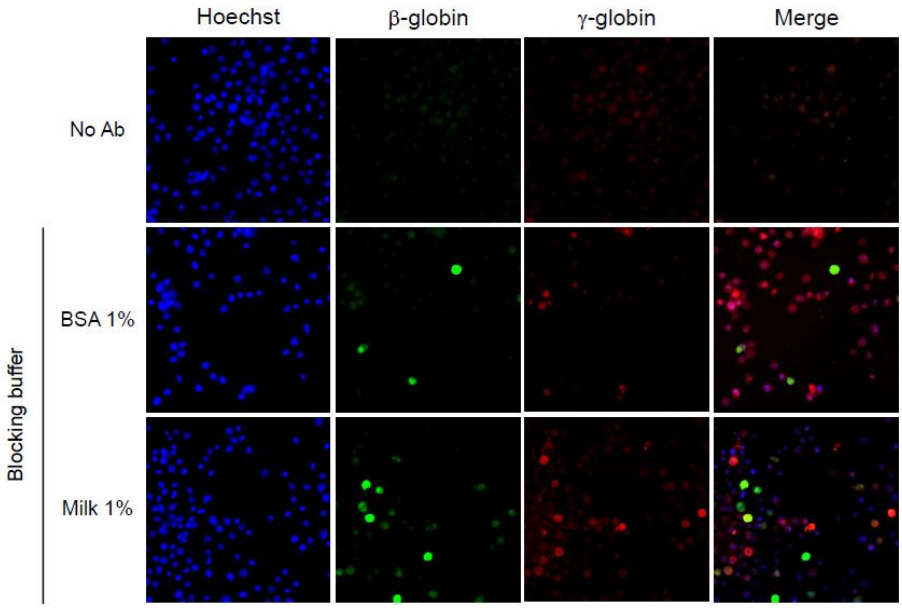


Figure 7 Representative data of conditions setup for high-content analysis in β-K562

Cells were stained with nuclear marker (Hoechst 33342), anti-γ-globin-PE and anti-β-globin-FITC antibodies, in different blocking buffers, as 1%BSA and 1% of milk. The most suitable conditions were obtained after overnight staining with 1% of milk

2.2. Readout and data analysis

K562 and β-K562 cells were stained with the DNA dye Hoechst 33342 and immunostained by specific anti-γ-globin-PE and anti-β-globin-FITC antibodies. Cells were analyzed with an ArrayScan VTI high-content screening reader and fluorescence images were acquired and processed as shown in Fig 9 to obtain quantitative fluorescence imaging readout at the single cell level. Cell recognition/count and nuclear parameters (DNA content, morphology and area) were obtained from the Hoechst signal in the Channel 1 (Ch1). Fluorescence intensity of β-globin (FITC-conjugated) and γ-globin (PE-conjugated) were respectively measured in the Channel 2 (Ch2, green fluorescence) and the Channel 3 (Ch3, red fluorescence).

Control isotypes (IgG-FITC and IgG-PE) were employed as references to adjust the exposure times in the different fluorescence channels and to reduce bleed-through of fluorescence emission across channels: this procedure resulted in optimal separation of the fluorescence emission in the individual channels (Fig 8).

To set up the correct threshold for every channel separately, staining applying isotypes (IgG-FITC and IgG-PE) controls has been performed.

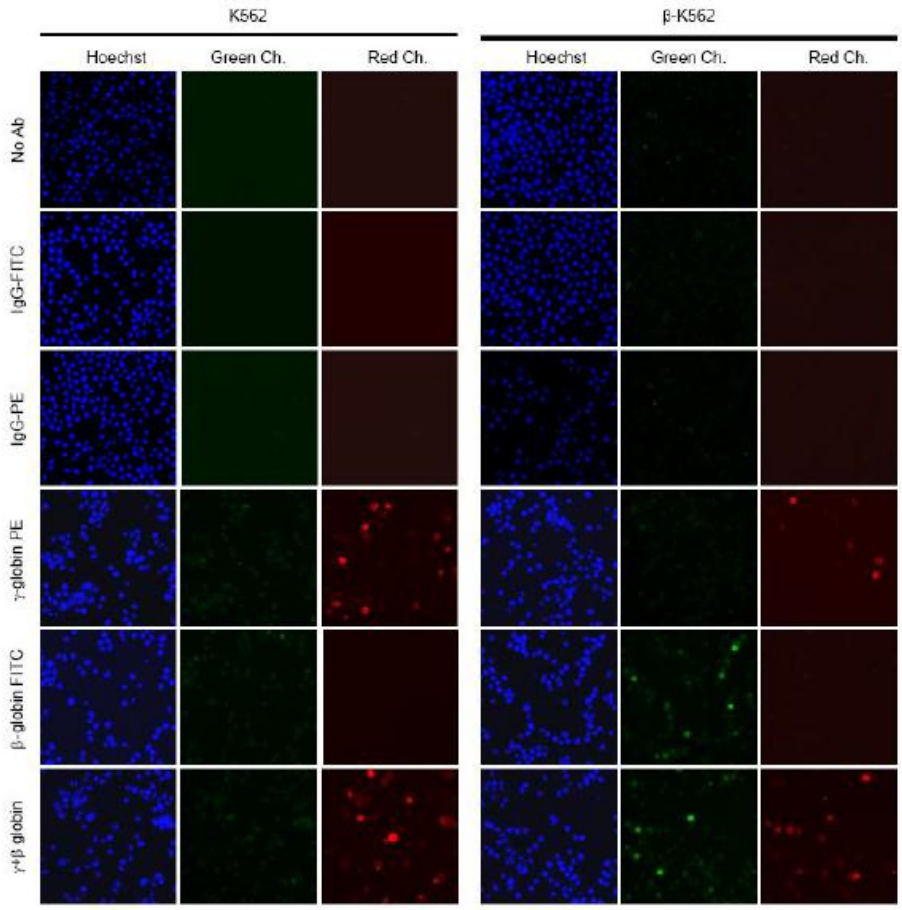


Figure 8 Isotopic controls analysis of K562 and β-K562
Cells were stained with antibodies anti- IgG-FITC, IgG-PE, γ-globin (PE), β-globin (FITC), and with anti-γ/β. Well separated signal in every channel confirmed suitability of the assay.

Based on their nuclear staining, cell nuclei were recognized and counted as objects in Ch1 and subsequently associated to two parameters: Nuclear Area and Nuclear Intensity (corresponding to DNA content). Enucleated cells were thus excluded from analysis. After object identification, the image analysis algorithm measures the cytoplasmatic fluorescence intensity (MFI, Mean Fluorescent Intensity) associated to hemoglobin content in every single cell (Ch2 for β-globin-FITC and Ch3 for γ-globin-PE).

The fluorescence signal in three channels is shown for six cells as an example: cell 1, 2 and 3 (Fig 9B) are double negatives (γ−β−); cell 4 is single β positive (γ−β+); cell 5 is single γ positive (γ+β−); cell 6 is double positive (γ+β+). Double expression of γ plus β results in an orange/yellow coloration (merging of green and red) of different intensity, depending on the relative expression of γ and β globins (see cells 3 and 6). Applying a Microsoft Excel macro developed in house, we calculated the percentage of single-positive (γ+ or β+), double positive (γ+β+) or double negative (γ−β−) cells in a population. At least 500 cells were acquired and analyzed for each sample and single-cell globin fluorescence signals plotted as in Fig. 9C.

A representative experiment (n=5) using K562 (ECACC) and β-K562 shows the heterogeneous distribution of control cell populations: untreated, parental K562 cells are mostly γ+β− (54.6% ± 3.7%), with a few β-positive cells (3.7% ± 0.4%). On the contrary, β−K562 cell line is composed of about 55% γ-globin positive cells (40.5% γ+β- ± 14%γ+β+) and about 17% of β-positive cells (14% γ+β+ ± 2.7% γ−β+). Interestingly, the majority of β-positive β-K562

cells co-expresses γ-globin (around 14% of cells), whereas only few appears to be exclusively β-positive. Overall, these data confirm that β-K562 express β-globin, as shown by flow cytometry, qRT-PCR and confocal microscopic analysis, and provide additional information on population heterogeneity.

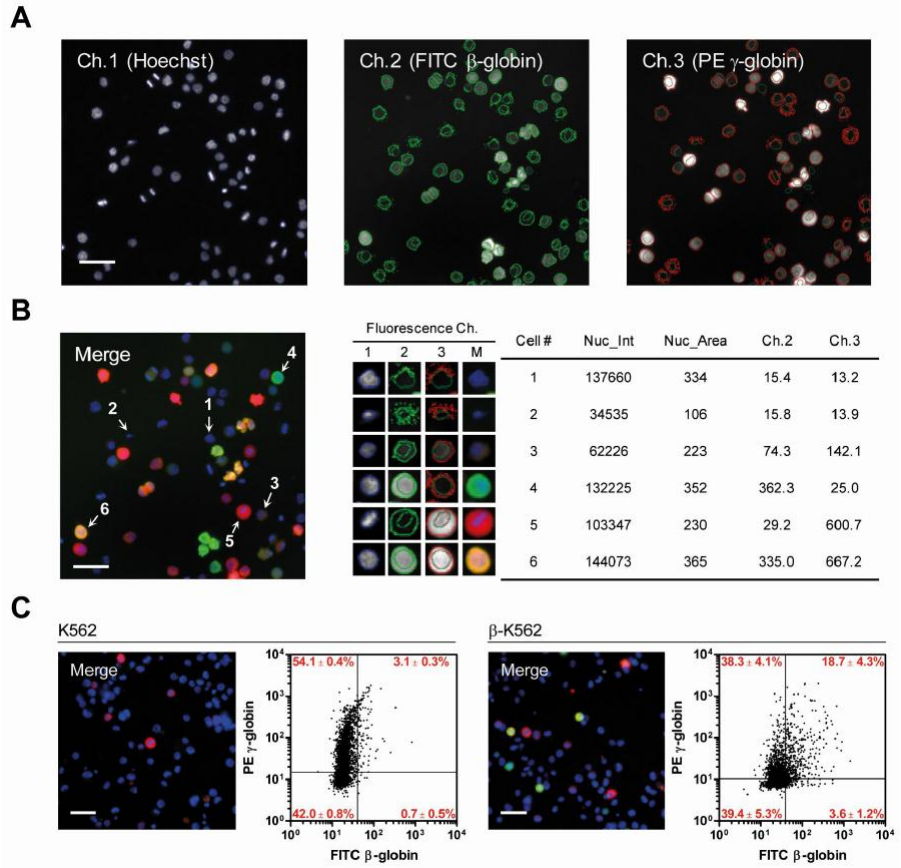


Figure 9 Analysis of b/b globin levels by immunofluorescence and automated image capture

A) Image acquisition and analysis. Nuclei were stained with Hoechst-33342; HbF and HbA were immunostained by using the specific anti γ- and anti β-globin antibodies. The intensity value of signals is automatically assigned by the instrument (Table in panel B) and converted into the corresponding intensity of colors: blue for Hoechst (Ch1), green for β-globin (Ch2) and red for γ-globin (Ch3), respectively. B) Images acquired in the single channels (Ch1, Ch2, Ch3) are merged, as shown in the left panel (Merge). Quantitative fluorescence imaging of single cells (right panel). Cells numbered from 1 to 6 in the Merge panel are taken as an example of γ–β– double negative (1 and 2), single γ+β- positive (5), single γ-β + positive (4) and γ+

double positive (3 and 6). C) "Merge" image and plot of K562 (left panels) and β-K562 (right panel), confirming a significant expression of β-globin in these latter cells (n≥3). Bar=50 μm.

2.3. Assay validation with known hemoglobin inducers

To test the sensitivity of the method in the detection of changes in levels of γ and β globins, we treated β-K562 cells with known hemoglobin inducers like Hydroxyurea (HU) and Butyric acid (BA). Dose-response curves were performed using the ArrayScan HCS reader for protein levels and RT-PCR for mRNA levels. Compound treatments resulted in increase of hemoglobin content in cells, and reduction of the percentage of double negative cells from 37.7% (SD=3.7) for controls to 18.1 (SD=10.9) for Hydroxyurea 800 μM and 15.75 (SD=6.4) for Butyric Acid 900 μM. Mean Fluorescence Intensity (MFI) associated to γ-globin immunofluorescence increased from 72.7 in untreated cells to 178.0 and 125.9 for HU and BA respectively. Similarly β-globin MFI increased from 209.8 (controls) to 238.6 (HU) and 218.4 (BA). As shown in Fig 10. BA treatment preferentially induces γ-globin expression, whereas Hydroxyurea stimulates both γ- and β-globins, resulting in an increase of double positive ($\beta^+\gamma^+$) cells. RT-PCR analysis has been performed in parallel on samples treated with the same conditions- both compounds greatly induces γ– globin mRNA expression with respect to untreated cells, whereas β-globin is slightly increased. Modest (2 to 4-fold) changes in the levels of α-globin were observed following treatment with both compounds. Overall, RT-PCR data confirm the strong effects of HU and BA on γ-globin induction observed at the protein level by high-content analysis.

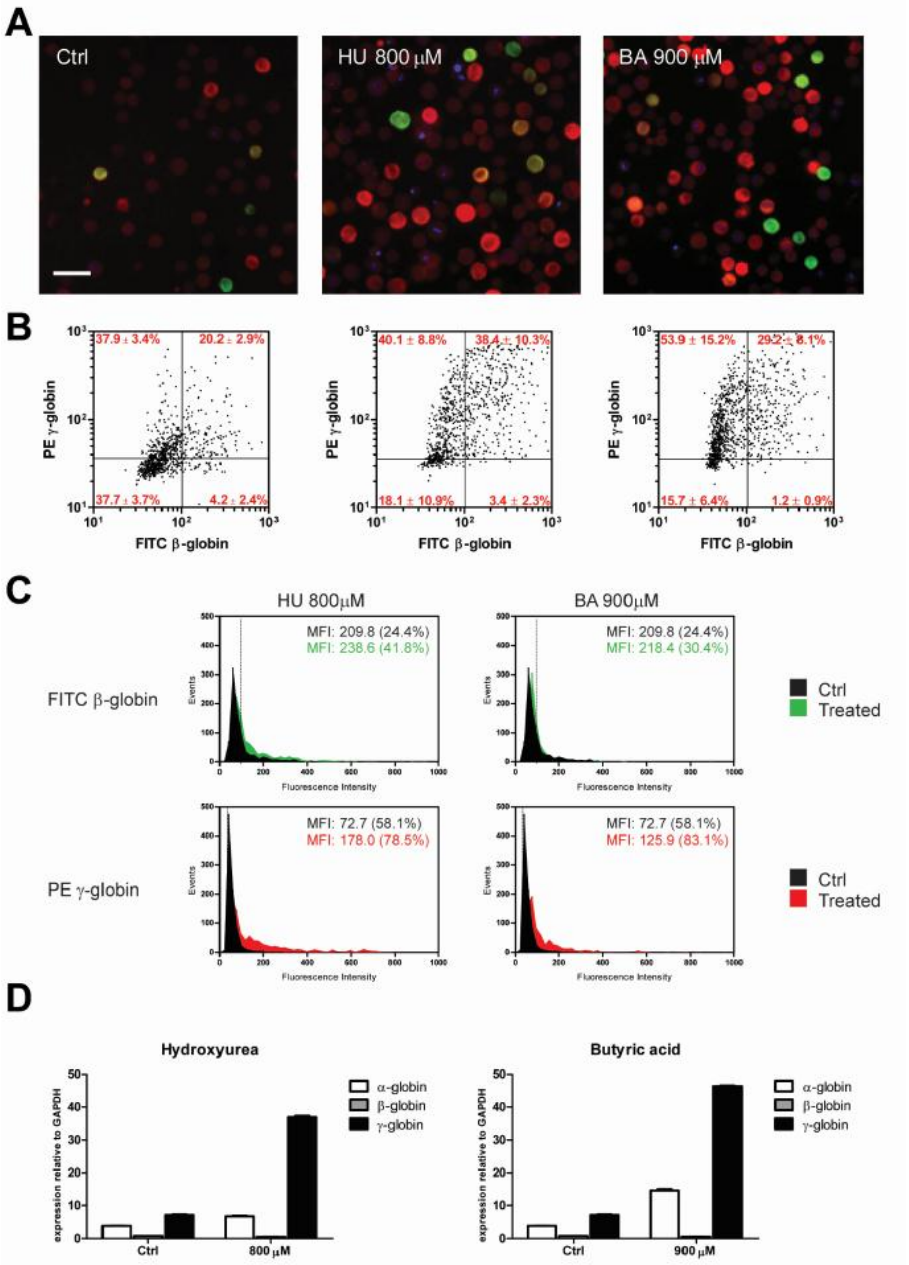


Figure 10 High-content analysis of compound induced changes in globin accumulation

β-K562 cells were treated with 800μM Hydroxyurea and 900μM Butyric Acid (n=3, a representative experiment is shown) and the same cells were analyzed in parallel by immunofluorescence and by RTqPCR 4 days after the addition of the drugs. A) Immunofluorescence images. Bar=50μm. B) Scatter plots giving the percentage of double γ–β– negative, single γ+β- positive, single γ–β+ positive and double γ+β+

positive cells (x axis: FITC-β-globin; y axis: PE-γ-globin). C) Fluorescence intensity plot to better visualize the changes in mean fluorescence intensity (MFI) of stained cells upon drugs treatment. Y axis: number of events (cells); X axis: fluorescence intensity for β globin signal (upper panels) or γ globin signal (lower panels), respectively. Green/Red curves: treated cells. Black curve: untreated cells. The vertical dotted line within each panel corresponds to the threshold set in panel B. The MFI and the percentage of positive cells (%) are indicated within each panel. D) RTqPCR on α, γ and β globins. Histograms show the relative levels of expression relative to GAPDH.

Butyric acid and Hydroxyurea are well known differentiating agents for hematopoietic cells. To confirm induction of β-K562 erythroid differentiation by compound treatment with an independent marker, in addition to the increase of globin expression, cells were analyzed by CD235a (Glicophoryn A) immunofluorescence, that is a membrane marker frequently employed to assess erythroid differentiation Fig. 11. β-K562 cells were thus stained with Hoechst and immunostained with three antibodies (anti-γ-globin-PE, anti–β-globin-FITC and anti-CD235a-APC), resulting in a four-channels high-content assay.This analysis confirmed differentiation after stimulation, but also the suitability of the approach to perform multiparametric analysis.

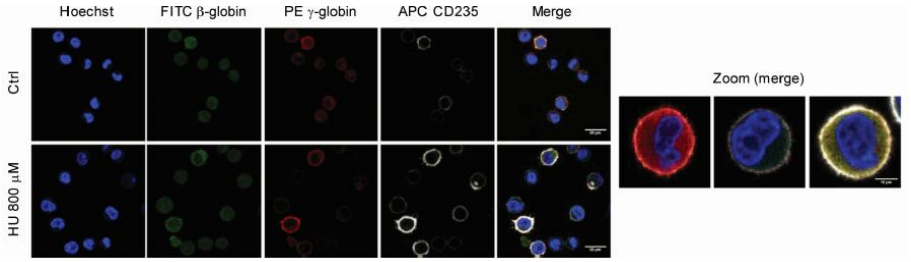


Figure 11 Confocal analysis of b-K562 cells, untreated or treated with HU
A quadruple staining with Hoechst (blue), anti β- (green), anti γ-globin (red) and anti-CD235a (white). Magnification: 20x. Right panel: 40x magnification of individual cells γ+CD235a+or β+CD235a+ double positive and γ+β+CD235a+ triple positive, respectively.

3. High content siRNA screening in β-K562 to identify modulators of hemoglobin synthesis

Experimental conditions previously established for efficient siRNA delivery into K562 cells were confirmed as suitable also for β-K562 (Fig. 12). Once confident in the reliability of transfection, a high-content siRNA screening was performed using a set of oligos targeting genes related to hemoglobin transcriptional regulation and metabolism, for a total 177 siRNA against 77 genes (at least 2 siRNA oligos per gene).

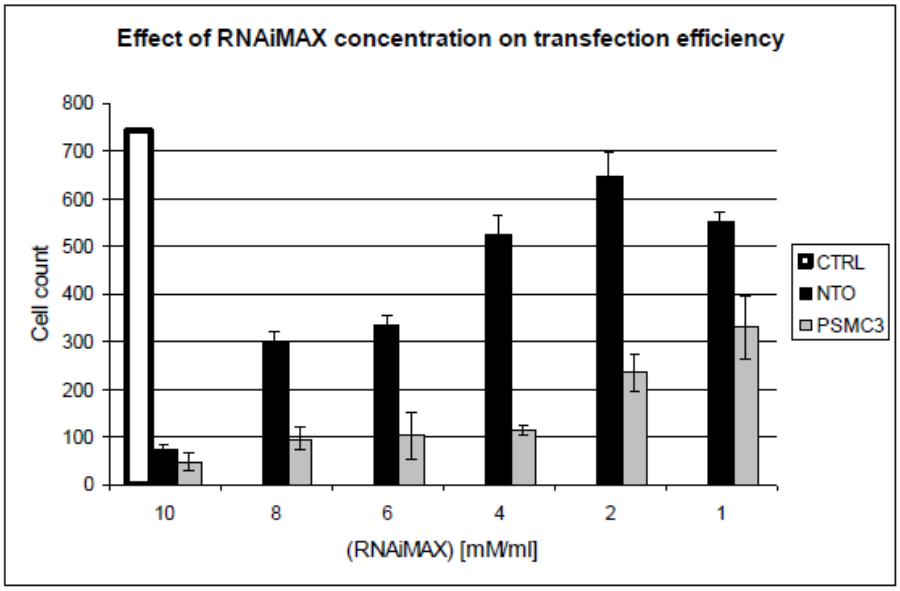


Figure 12 Dose-response curve of RNAiMAX concentration effect on a cell count in b-K562 cell lines
One hundred cells were transfected with 20nM siRNA oligos and incubated for 7 days, then fixed and counted. Experiment showed that conditions that had been established for K562 (ECACC) could be applied also for β-K562.

For any sample, proliferation data were expressed as the mean cell count per field normalised on the average cell count of NTO controls within the same plate. Similarly, β and γ-globin fluorescence

intensities were normalised in the respective channels with respect to the average MFIs of NTO controls.

The transfection efficiency was assessed by measuring the antiproliferative effect of control siRNA oligonucleotides targeting PSMC3 and Eg5, two genes which regulates fundamental cellular processes (proteasome function and cytokinesis, respectively) and whose silencing is expected to result in cell death.

Proliferation results confirmed that the conditions set up ensured optimal efficiency of transfection: siNTO: 100.8% ± 4.2; siPSMC3: 22.6% ± 14.5, siEg5: 4.8% ± 3.1. Moreover, siRNA silencing of γ–globin (siHbG) and β globin (siHbB) resulted in almost complete disappearance of their respective immunofluorescence signals as demonstrated in Fig. 13.

The siRNA screening allowed identification of genes with a potential role in modulating hemoglobin levels, thus representing putative targets for targeted therapy of β-thalassemia. Heme-oxygenase 2 HMOX2, but not Heme-oxygenase 1 (HMOX1) was shown to strikingly induce globin level, with prevalent accumulation of γ-globin (Fig. 13A). At least two HMOX2 siRNA oligos induced protein levels of γ– and β–globin, both in terms of increase in number of positively expressing cells, as well as in terms of MFI of globin signal per cell.

Notably, HMOX2 did not affect cell proliferation and morphology. HMOX2 is an enzyme responsible for hemoglobin cleavage into biliverdin (further decomposed to bilirubin by biliverdin reductase) iron and carbon monoxide. HMOX1 is an inducible gene,

whereas HMOX2 is constitutively expressed and exclusively expressed at high levels in K-562 cell line (Ding Y., *et al*. (2006) .

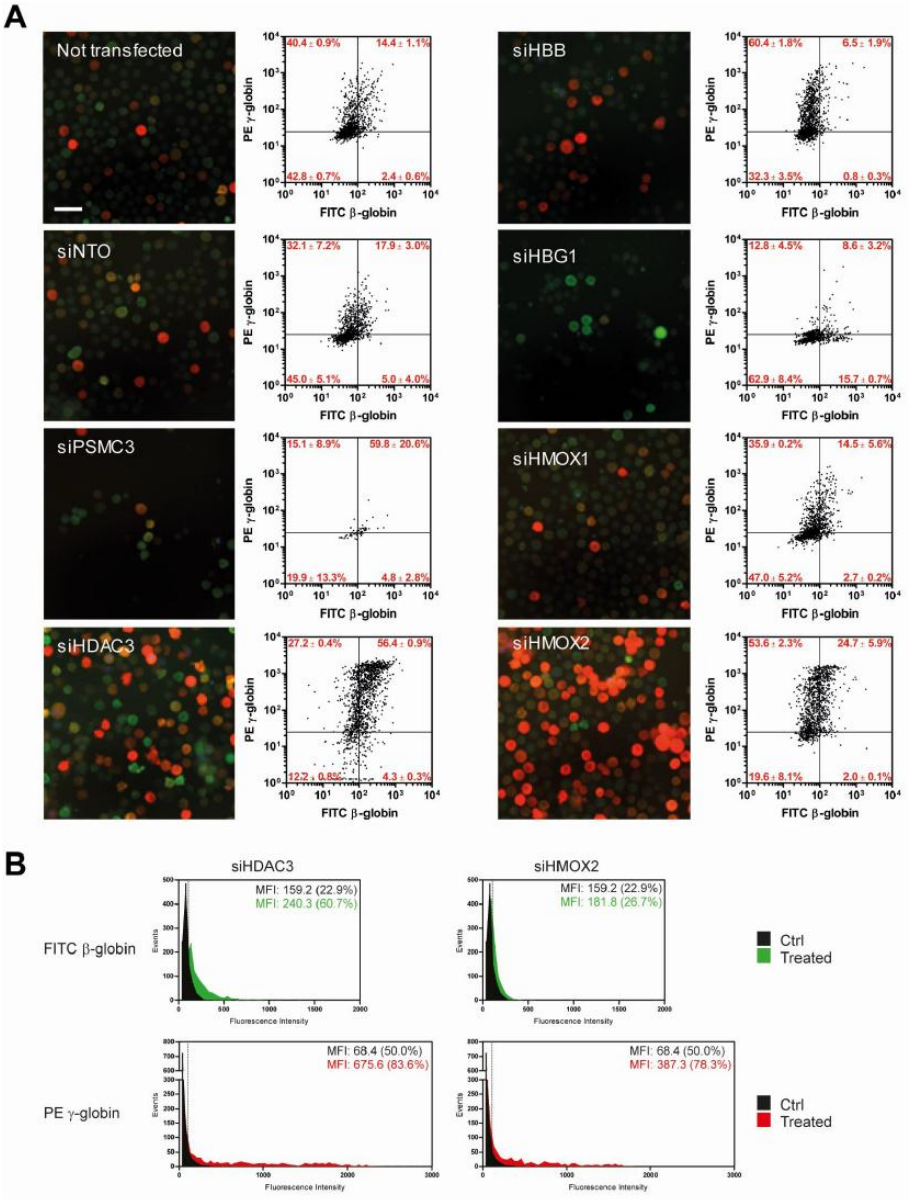


Figure 13 High-content g/b-globin analysis as readout of siRNA screening in b-K562 allows identification of novel genes affecting hemoglobin levels.
A) Cells were transfected with a non-targeting oligo (siNTO) as negative control and with a siRNA directed to PSMC3 as positive transfection control. As further control, siRNAs targeting γ and β globins greatly reduced the corresponding globin chains.

For each gene, three siRNA were tested, with two technical replicates (immunofluorescence images of representative experiments are shown). Bar=50μm. Scatter plots are provided for each immunofluorescence image (n=2). B) Fluorescence intensity plots relative to siHDAC3 and siHMOX2.

To confirm HMOX2 effect on hemoglobinization we treated β-K562 with increased doses of a selective inhibitor of heme-oxygenases. Tin protoporphyrin IX (Tin-PPIX) (Frank J., *et al*. 2007), in comparison with hemin, which is known to raise globin levels in erythroleukemic cells (Ross J, Sautner D., 1976). Compound treatments confirmed a dose-dependent induction of γ-globin as percentage of positively stained cells and, as MFI increase. Interestingly, whereas hemin treatment significantly increases the percentage of double γ/β positive cells, Tin-PPIX seems to have a more selective activity on γ-globin, as confirmed by the MFI values. A similar effect was observed for mRNA levels, analyzed by RT-PCR: both compounds increased γ–globin mRNA levels, a suggesting transcriptional regulation mechanism.

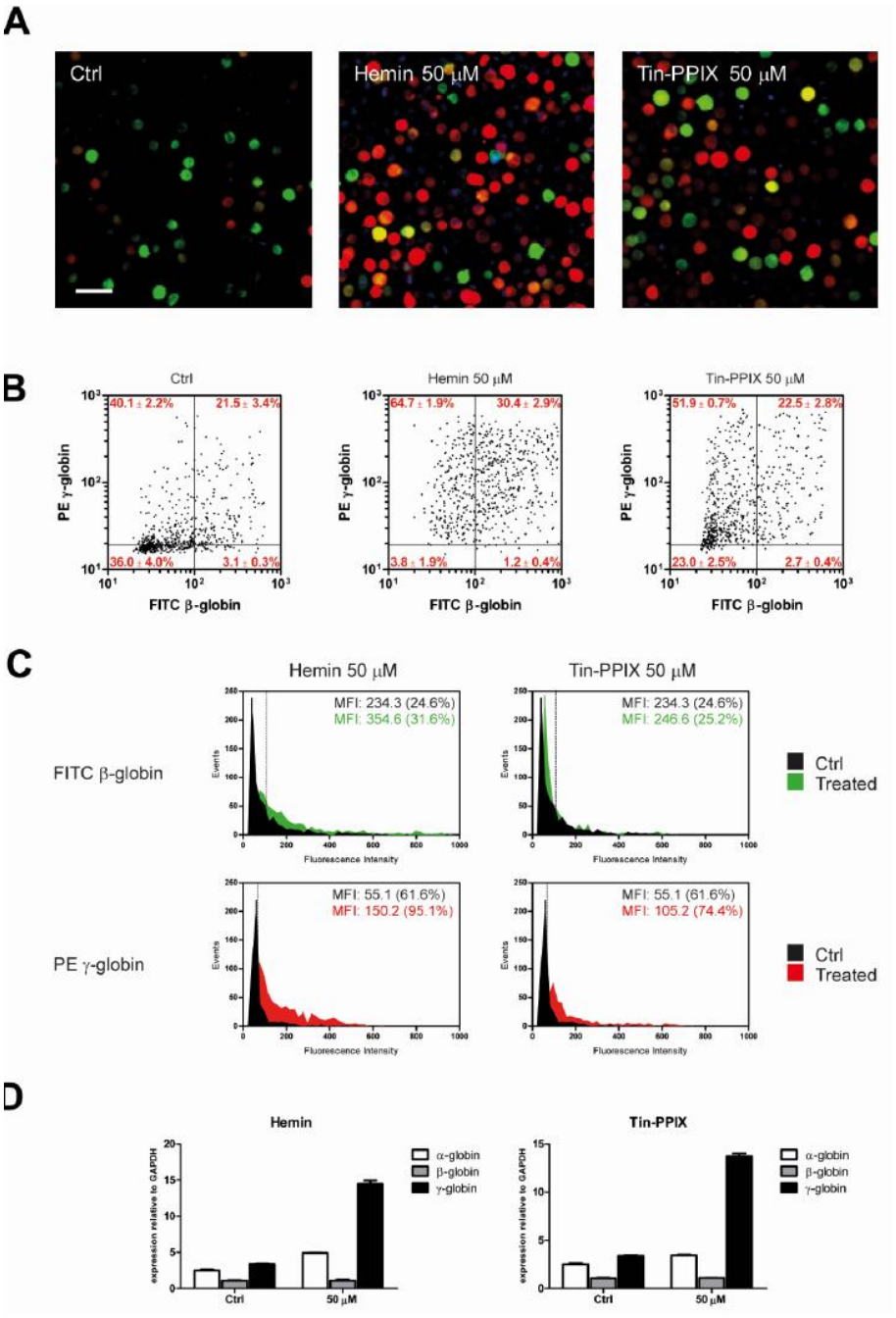


Figure 14 Hemin and Tin-PPIX have similar effects on β-K562 hemoglobinization levels

Cells were treated with 50μM of either Hemin or Tin-PPIX for four days and then analyzed as in Fig.3 (n=3, a representative experiment is shown). A)

Immunofluorescence images. Bar=50μm. B) Scatter plots. C) Fluorescence intensity plot. D) RTqPCR on α, γ and β globins. Histograms show levels of globins expression relative to GAPDH.

The analysis of normalised data was performed using SpotFire software (Fig 15). Based on their phenotypic effect on proliferation and hemoglobinization of β-K562 cell line, siRNA oligos were divided into three categories:

1. oligos that inhibit cell proliferation associated to increased globin levels, thus potentially inducing β-K562 terminal differentiation (cell count <30 %NTO and γ-globin MFI > 150);

2. oligos that induce cell death (cell count <30 %NTO and γ-globin MFI < 150);

3. oligos that increase globin levels, with no effects on cell proliferation (cell count >80 %NTO? and γ-globin MFI > 150).

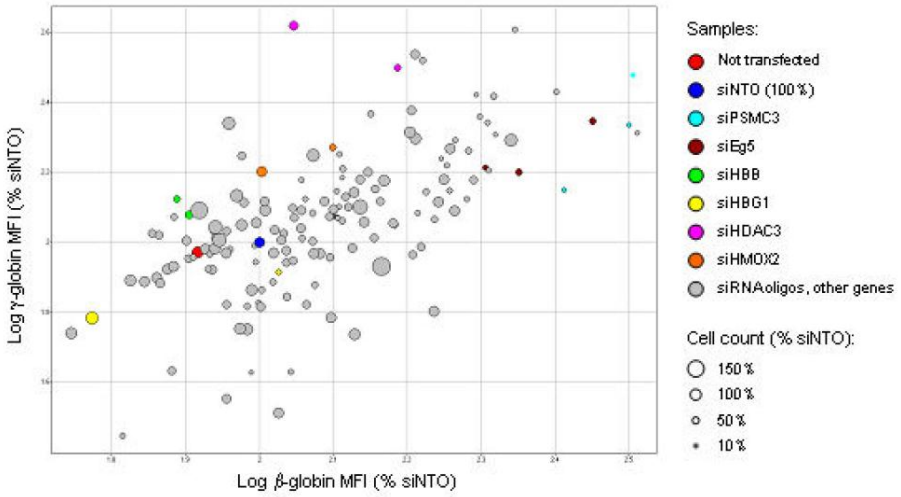


Figure 15 Scatter plot of β/γ–globin mean fluorescence intensity (MFI) expressed as logarithmic value after normalisation on NTO (100%; x axis: FITC-β-globin; y axis: PE-γ-globin). Selected siRNA oligonucleotides are highlighted with different colors. The size of each dot represents the cell number in a given sample (cell count per field from ArrayScan analysis), normalised on the siNTO (100%).

siRNA oligonucleotides belonging to the first group of hits include, among others, the ones targeting EIF4A1 and EIF4G1, with at least 2 oligos with different sequence inducing the same phenotype (Fig 16B). Eukaryotic Translation Initiation Factor 4 Gamma 1 (eIF4G1) and Eukaryotic Translation Initiation Factor 4A1 (eIF4A1) belong to eIF4F multiprotein complex that promotes the interaction between mRNA five-prime cap and ribosomes. eIF4A protein is essential for cell vitality as involved in translation initiation: it removes 5'-untranslated region of mRNA facilitating ribosomal binding. Literature data demonstrate the dramatic effect of eIF4A1 silencing on cell viability in diverse cell lines, especially those actively proliferating (Galicia-Vázquez G., *et al.*, 2012). EIF4G1 is a scaffold protein that directly interacts with eIF4A1 to compose part of the translation initiation complex (Li W., *et al.*, 2001).

The siRNA oligos belonging to the second group decreased proliferation without inducing hemoglobinization: in this group we found oligos targeting the genes BCL2L14, HBG1, HBS1L and SRSP1 (Fig 16C).

BCL2L14 (apoptosis facilitator BCL2-like, alias Bcl-G) is cytoplasmic protein with multiple functions, including protein kinase binding. BCL2L14 belongs to the BCL2 family of pro-apoptotic function and it is expressed as two isoforms: a long isoform (Bcl-GL) and a short one (Bcl-GS). Whereas Bcl-GS is associated with induction of apoptosis by interaction with other members of the BCL2 family, Bcl-GL has no pro-apoptotic function due to lack of binding activity. The details about BCL2L14 function are still unclear (Tischner D., *et al.*, 2012).

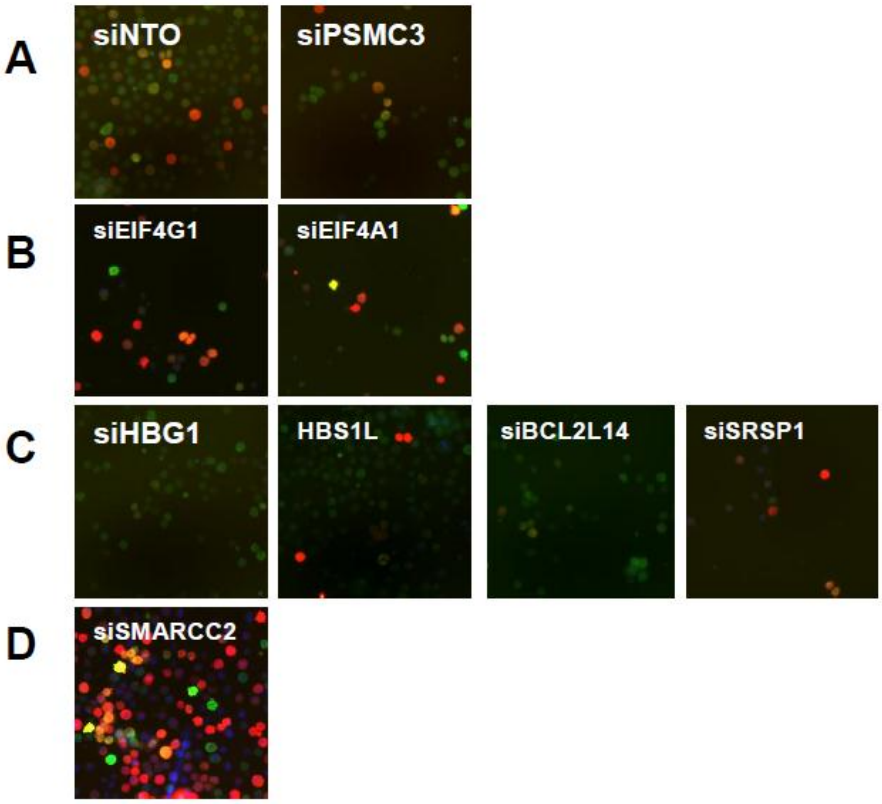


Figure 16 representative phenotype observed in siRNA transfected b-K562 cells A) controls; B) oligos that inhibit cell proliferation associated to increased globin levels, C) oligos that induce cell death D) oligos that increase globin levels, with no effects on cell proliferation.

The protein product of SRSP1 gene, alias SPATS1 (Spermatogenesis Associated, Serine-Rich 1), is detected in embryonic testis cells and has still unknown function.

HBG1 (Hemoglobin Gamma A) is the gene encoding for γ-globin chain, physiologically expressed during fetal development, that is replaced by the β-globin chain in adults. A pathological condition called Hereditary Persistence of Fetal Hemoglobin (HPFH) consists in the presence of elevated γ-globin levels in the adult. K562 and β-K562

cell lines express high levels of fetal hemoglobin, as demonstrated previously.

HBS1L (HBS1-like) gene encodes for a GTP-binding elongation factor family member. The intergenic region of this gene has been demonstrated as to be a quantitative trait locus (QTL), which regulates fetal hemoglobin levels. Several polymorphisms of HBS1L intergenic region have been associated with disease severity and painful crises in sickle cell anemia and β-thalassemia patients (Pereira C., et al., 2015, Thein SL., et al., 2007).

Epigenetic aberrations are known to be key drivers of several diseases like cancers, inflammation, cardiac and neurological disease (Falkenberg, *et al.*, 2014). Epigenetic modifications cause chromatin remodeling and aberran gene expression, that eventually lead to phenotypic changes. Epigenetic modifications my occur on DNA (e.g. DNA methylation) or chromatin proteins, mainly histones and include acetylation, phosphorylation and ubiquitylation. A third group of epigenetic changes are those associated with chromatin remodelling complexes, as NuRD or SWI/SNF (Falkenberg, *et al.*, 2014). Since epigenetic changes are reversible and chromatin remodeling enzymes are druggable by small molecules, we include in our screening siRNA oligonucleotides targeting this class of enzymes in order to identify any potential target for the treatment of β-thalassemia and SCA. The silencing effects were evaluated for SMARCC2:

SMARCC2 (alias BAF170, SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily C, Member 2) is a member of SWI/SNF chromatin remodeling complex. The protein encoded by this gene is an ATPase/DNA helicase which

regulates chromatin structure and influences gene expression as a consequence. Knockdown of SWI/SNF complex members in mouse models has been shown to be associated with delayed hemoglobin switching from fetal to adult (O'Neil, *et al.*, 1999). β-K562 cells were transfected with three siRNA oligos for SMARCC2 with different sequences, in duplicate: gene silencing resulted in strong γ-globin induction without relevant effects on cell proliferation.

We evaluated the effect of different HDAC (histone deacetylases) small molecule inhibitors on the β and γ globin expression in β-K562 cells.

HDACs are a group of enzymes, which remove acetyl residues from lysine and are currently exploited as therapeutic targets for a number of diseases including cancer, inflammation, neurological diseases and immune disorders (Flankenberg KJ, *et al.*, 2014), Around 500 clinical trials involving HDAC inhibitors have been performed up to now. Histone acetylation and de-acetylation cause important changes of DNA structure and controls gene expression: chromatin remodeling influences the accessibility of transcription factors to their targets genes. It has been estimated that treatment with HDAC inhibitors may change the expression of around 2-10% of genes.

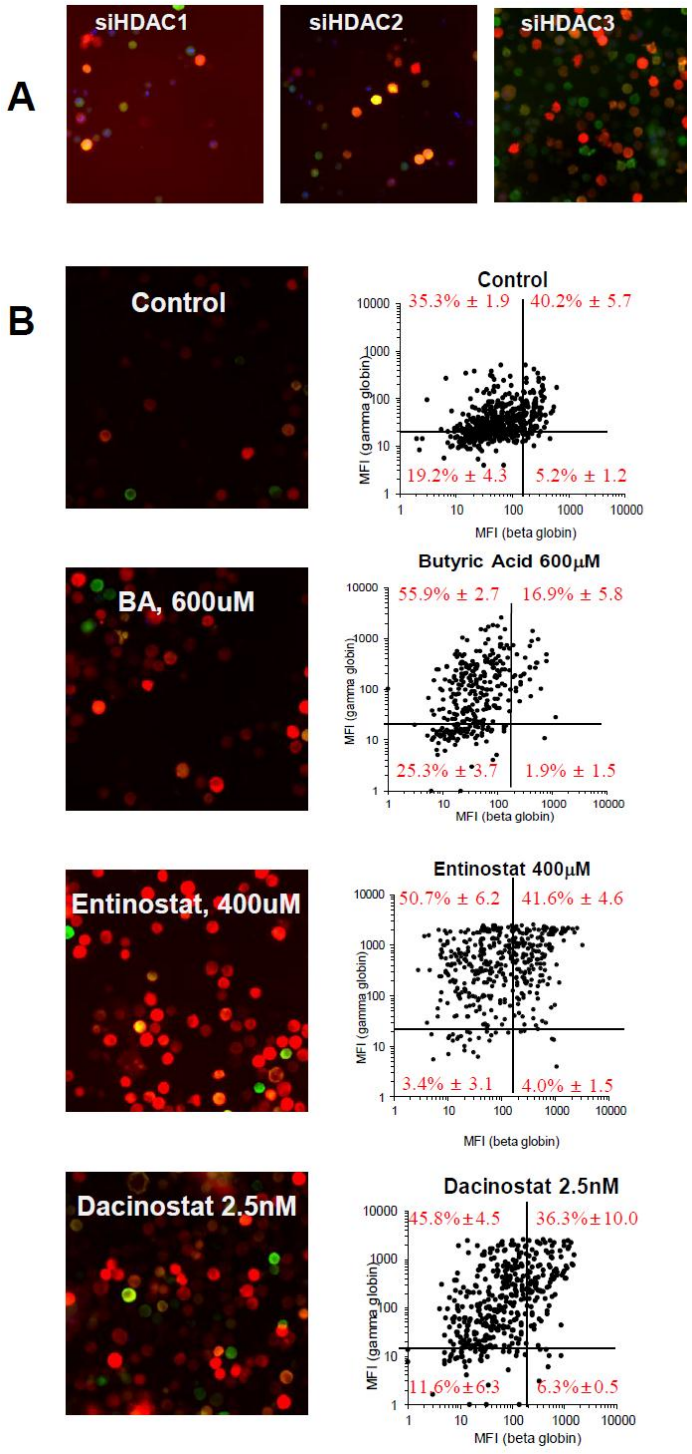


Figure 17 Representative data of HDAC inhibition on level of g and b-globin

A) siRNA knockdown of HDAC1, HDAC2 and HDAC3, B) HDAC inhibitors with corresponding scatter-plots.

Knockdown of the histone deacetylase 3 enzymes (HDAC3) is known to mediates γ-globin promoter de-repression, resulting in a strong induction of γ-globin levels (Bradner JE., et al., 2010). We treated our cell line model β-K562 with three HDAC inhibitors [butyric acid (BA), entinostat (MS-275) and dacinostat (LAQ-824)] in order to study their effect on the reciprocal expression of β and γ globin chains. Dacinostat has been considered as a therapeutic agent for myeloid leukemia since it promotes apoptosis in CML and AML cell lines at nanomolar concentrations and prolongs the overall survival time in a pre-clinical murine leukemia model (Weisberg E, *et al.*, 2004). Entinostat, an inhibitor of HDAC I and III, is a potent inducer of cell differentiation in AML cell lines, not associated with apoptosis induction (Blagitko-Dorfs N., *et al.*, 2013). Entinostat has been considered in myeloid leukemic patients, as well as a potential therapeutic agent for myelodysplastic syndromes (Blagitko-Dorfs N., *et al.*, 2013, Maggio SC., *et al.*, 2004). As expected all three compounds induced γ-globin accumulation in a dose-dependent manner. In particular, entinostat increased γ-globin positive cells from 54.5% (in untreated controls) to 92.5% and β-globin positive cells from 24.4% to 45.8%. A strong effect on globin expression was observed also with dacinostat (82.1 % of γ-globin and 42.5 % of β-globin positive cells) and butyric acid (72.8 % of γ-globin and 18.8 % of β-globin positive cells). These results confirm previous observations that BA is a strong inducers of γ-globin expression, but its efficacy on β-globin synthesis is limited. Moreover, we observed

that dacinostat does higher than 5 nM result in strong proliferation inhibition and cell death.

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Chapter 4

SUMMARY AND C LINICAL RELEVANCE

The aim of our study was to establish a novel siRNA transfection approach for large-scale screening in leukemic cell lines. We used the chronic myeloid cell line K562 as reference model to setup transfection procedure of efficient and nontoxic siRNA delivery in 96-well plates. Using control siRNA oligos (one non targeting oligo and two siRNA oligos inducing cell death, Eg5 and PSMC3), we tested different types of liposomic transfection reagents, different concentrations of siRNA oligonucleotides, different cell densities and plate geometries. The optimal conditions were the following: Lipofectamine RNAiMAX transfection reagent at a concentration of 4-6 μl/ml in the initial mixture; 10-20 nM siRNA oligo final concentration; cell density in the range 100-200 per well and incubation time of 7-10 days. The whole process is performed in U-bottom plates, then cells are transferred to flat bottom plates for analysis.

A pilot screening, performed to validate the transfection conditions, showed that established conditions were suitable for large-scale screening. Importantly, within the group of active oligos we found siRNA oligos targeting genes known to be required for K562 viability, like PLK1 and ABL1 (Luo J, et al., 2009).

High-content siRNA screening was performed using 5808 siRNA oligos targeting 1936 genes. Transfection efficiency was 91.09 ± 6.0. Among "top scoring" hits, we identified DNA and RNA polymerases, small nuclear ribonucleoproteins and kinases like

(ABL1, GAK, HCK or PLK1). Most of these genes have been already reported as essential in leukemic cell lines (Bhinder B., 2013, Luo J, et al., 2009). Technical replication of 32 active oligos identified 11 genes, as novel potential targets for drug discovery process. Further experiments are needed to investigate the role of these genes in promoting K562 proliferation and to validate them as potential targets to develop targeted therapies for erythroleukemias.

We developed a novel immunofluorescence-based multiparametric high-content assay by exploiting a K562 clone, that we named β-K562. DNA fingerprint analysis confirmed that K562 and β-K562 share the same clonal origin; these two cell lines only differ in hemoglobin content. Indeed, K562 cells show a fetal pattern, typical of erythroleukemic cells: they express embryonic (HbE, $\alpha 2\varepsilon 2$) and fetal (HbF, $\alpha 2\gamma 2$) hemoglobin, whereas β-K562 cells express fetal (HbF, $\alpha 2\gamma 2$) and adult hemoglobin (HbB, $\alpha 2\beta 2$). We set a procedure for β and γ-globin immunofluorescence combined with DNA staining (Hoechst 33342) for cell count and single-cell analysis of nuclear morphology and DNA content. To validate our assay, we treated β-K562 with known inducers of γ-globin: hydroxyurea and butyric acid: the assay resulted robust and suitable to study fetal and adult hemoglobin levels in an erythroleukemic cell line representative of human erythrocyte precursor cells.

We applied the same transfection conditions optimized for K562 cells to transfect the β-K562 cell line with 177 siRNA oligos (corresponding to 77 genes, with at least 2 siRNA oligos per gene) searching for modulators of globin levels and cell proliferation. The screening revealed new, potential molecular targets whose inhibition

resulted in increased γ-globin expression. In particular, among the hits we identified Heme-oxygenase 2 (HMOX2), a constitutively expressed enzyme responsible for heme degradation (Ding Y., *et al*. 2006). HMOX2 siRNA silencing resulted in a strong increased levels of γ–globin and β–globin in cells, without affecting cell count or inducing any morphological changes of cells. To validate HMOX2 inhibition as a potential target for the treatment of β-globinopathies (i.e., Sickle Cell Disease and β-thalassemia), we treated β-K562 with Tin protoporphyrin IX (Tin-PPIX), a selective HMOX inhibitor, in comparison with hemin, a known inducer of γ-globin (Ross J, Sautner D., 1976). Tin-PPIX preferentially induced the γ-globin levels, whereas hemin increased both β and γ-globin levels. Results were confirmed by RT-PCR, indicating that the drugs regulate globin genes at the transcriptional level. Interestingly, we found genes which knockdown resulted in hemoglobinisation of β-K562 cells accompanied (EIF4A1 and EIF4G1) or not (BCL2L14, HBG1, HBS1L and SRSP1) to inhibition of proliferation.

This evidence indicates that our assay is able to identify drugs or genes which promote globin expression accompanied by inhibition of proliferation, thus representing putative targets preventing terminal differentiation of K562. The genes belonging to this group can be further studied to identify molecular targets to treat erythroleukemias through induction of terminal differentiation. On the other hand, genes or drugs that have a transcriptional effect on globins expression without inhibition of cell proliferation, like HMOX2, can be identified as well using our assay. We speculate that this second group of targets includes modulators of globin chains expression or heme stability, like

HMOX2, that could be further studied to develop innovative therapies to treat β-hemoglobinopathies.

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ABBREVIATIONS

ABL1	Abelson Kinase 1
ACE-011	Sotatercept
ALL	Acute Lymphoblastic Leukemia
alloSCT	Allogeneic Stem Cell Transplantation
BA	Butyric Acid
BCL2L14	Apoptosis Facilitator BCL2-like, alias Bcl-G
BCL11A	Kruppel multi-zinc finger transcription factor
BMT	Bone marrow transplantation
CCyR	Complete Cytogenetic Response
ChIP	Chromatin Immunoprecipitation Assay
CML	Chronic Myeloid Leukemia
CMML	Chronic MyeloMonocytic Leukemia
COUP-TFII-	Chicken Ovalbumin Upstream Promoter- Transcription Factor
DNA	Deoxyribonucleic Acid
DOPE	1,2-Dioleoyl-sn-Glycero-3-Phosphoethanolamione
DOPC	1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine [C44H84NO8P])
DOTAP	2-dioleoyl-3-trimethylammonium-propane (chloride salt) [C42H80NO4Cl])
eIF4A1	Eukaryotic Translation Initiation Factor 4A1
eIF4G1	Eukaryotic Translation Initiation Factor 4 Gamma 1
ELN	European Leukemia Net
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate

GAK	(auxilin 2) Cyclin G-associated Kinase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HbB	Adult Hemoglobin
HbF	Fetal Hemoglobin
HbS	S Hemoglobin
HCK	Hematopoietic Cell Kinase
HDAC	Hhistone Deacetylase Inhibitor
HMOX1	Heme-oxygenase
HMOX2	Heme-oxygenase 2
HQK-1001	Sodium 2,2 dimethylbutyrate
HU	Hydroxyurea
IKBKB	Inhibitor of Kappa Light Polypeptide Gene Enhancer in B-Cells
KIF11/Eg5	Kinesin Family Member 11
KLF1	Erythroid Kruppel-like factor 1
LONP1	Lon peptidase 1
MCV	Mean Corpuscular Volume
MDS	MyeloDysplastic Syndromes
MFI	Mean Fluorescent Intensity
miRNA	microRNA
MMR	Major Molecular Response
NO	Nitric Oxide
NTO	Non Targetin Oligo
NUP98	Nucleoporin 98
PCyR	Partial Cytogenetic Response
PDGFR	Platelet-Derived Growth Factor Receptor
PE	PhycoErythrin

PITRM1	Pitrilysin Metallopeptidase 1
PLK1	Polo-Like Kinase 1,
PSMC3	Proteasome (prosome, macropain) 26S Subunit, ATPase, 3
RAN	G protein member of the Ras oncogene family
RANGAP1	Ran GTPase activating protein 1
RCC1	Regulator of Chromosome Condensation 1
RNAi	RNA Interference
RT-PCR	Reverse transcription polymerase chain reaction
SFKs	Src-family kinases
shRNA	short-hairpin RNA
siRNA	short interfering RNA
SCD	Sickle cell disease
SMARCC2	SWI/SNF Related, Matrix Associated, Actin Dependent Regulator Of Chromatin, Subfamily C, Member 2(alias BAF170,)
SOX6	SRY family of HMG box transcription factor
SRSP1	Spermatogenesis Associated, Serine-Rich 1 (alias SPATS1),
Tin-PPIX	Tin protoporphyrin IX
TKI	Tyrosine Kinase Inhibitor
USP39	Ubiquitin Specific Peptidase 39
WHO	World Health Organization

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