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Proteome changes induced by *c-myb* silencing in human chronic myeloid leukemia cells suggest molecular mechanisms and putative biomarkers of hematopoietic malignancies

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ABSTRACT

To shed light on the molecular mechanisms associated with aberrant accumulation of c-Myb in chronic myeloid leukemia, comparative proteomic analysis was performed on *c-myb* RNAi-specifically silenced K562 cells, sampled on a time-course basis. 2D-DIGE technology highlighted 37 differentially-represented proteins that were further characterized by nLC-ESI-LIT-MS/MS and validated by western blotting and qRT-PCR analysis. Most of the deregulated proteins were related to protein folding, energy/primary metabolism, transcription/translation regulation and oxidative stress response. Protein network analysis suggested that glycolysis, gluconeogenesis and protein ubiquitination biosynthesis pathways were highly represented, confirming also the pivotal role of c-Myc. A specific reduced representation was observed for glyceraldehyde-3-phosphate-dehydrogenase and α-enolase, suggesting a possible role of c-Myb in the activation of aerobic glycolysis. A reduced amount was also observed for stress responsive heat shock 70 kDa protein and 78 kDa glucose-regulated protein, previously identified as direct targets of c-Myb. Among over-represented proteins, worth mentioning is the chromatin modifier chromobox protein homolog 3 that contributes to silencing of *E2F*- and *Myc*-responsive genes in quiescent G_0 cells. Data here presented, while providing novel insights onto the molecular mechanisms underlying c-Myb activity, indicate potential protein biomarkers for monitoring the progression of chronic myeloid leukemia.

Biological significance
Myeloid leukemia is a malignant disease of the hematopoietic system in which cells of myeloid lineages accumulate to an undifferentiated state. In particular, it was shown that an aberrant accumulation of the c-Myb transcriptional factor is associated with the suppression of normal differentiation processes promoting the development of the hematopoietic malignancies. Many efforts have been recently made to identify novel genes directly targeted

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by c-Myb at a transcriptome level. In this work, we originally describe a differential proteomic approach to facilitate the comprehension of the regulation of the protein networks exerted by c-Myb. Our study reveals a complex network of proteins regulated by c-Myb. The functional heterogeneity of these proteins emphasizes the pleiotropic role of c-Myb as a regulator of genes that are crucial for energy production and stress response in leukemia. In fact, variations in glyceraldehyde-3-phosphate-dehydrogenase and α-enolase suggest a possible role of c-Myb in the activation of aerobic glycolysis. Moreover, significant differences were found for heat shock 70 kDa protein and 78 kDa glucose-regulated protein known as direct c-Myb targets. This work highlights potential protein biomarkers to look into disease progression and to develop translational medicine approaches in myeloid leukemia.

1. Introduction

The proto-oncogene *c-myb*, the cellular homologue of the transforming oncogene *v-myb* of avian myeloblastosis (AMV) and avian leukemia virus E26 [1], encodes a transcription factor that acts as a key regulator at different stages of hematopoiesis (*i.e.* in the proliferation and differentiation of hematopoietic precursors) [2]. In fact, it has been shown that low levels of c-Myb are required for terminal differentiation of myeloid and erythroid cell lines. On the contrary, aberrant high levels of c-Myb are associated with the suppression of normal differentiation processes promoting the development of several hematopoietic malignancies [3], T-cell leukemia [4], chronic myeloid leukemia (CML), acute myeloid leukemia [5,6], as well as other tumors, such as colorectal cancer [7], breast cancer [8] and adenoid cystic carcinomas [9]. In CML, aberrant high levels of c-Myb are required for *in vitro* proliferation and survival of leukemic progenitor cells through the blocking of the maturation-associated growth arrest. Indeed, it has been shown that c-Myb influences the G_2/M-phase cell cycle transition in human hematopoietic cells through the regulation of cyclin B1 [10] and is also required during the G_1/S-phase transition [11].

Being located on chromosome arm 6q in humans, *c-myb* gene is transcribed and translated into an abundant nuclear protein of 75 kDa with a known half-life of around 30 min [12] which operates mostly as a transcriptional activator, binding to a specific (t/cAACt/gG) nucleotide sequence, also known as MYB-binding site (MBS) [13]. Alternatively, spliced *c-myb* transcripts have also been characterized in human hematopoietic cells that generate less abundant gene products, such as $p89^{c\text{-}Mybex9b}$ [14]. Several studies have shown that c-Myb levels and activity can be finely regulated by post-translational modification (PTM)- [15,16], protein–protein interaction- [17,18] and mi-RNA-dependent mechanisms [19,20]. c-Myb can directly modulate the expression of many genes [2], among which some (*i.e. CD34*, *c-kit*, *c-myc*, *flt-3*, *Bcl-2*) exert important roles in the proliferation and survival of hematopoietic cells [21–23]. Recently, it has also been demonstrated that c-Myb can control the expression of the transcription factor Slug, which is essential for the homing of chronic myeloid leukemia cells within the bone marrow [24]. On this basis, c-Myb seems to be directly implicated in the *trans*-differentiation process known as epithelial mesenchymal transition (EMT), which frequently occurs at the onset of the metastatic cascade present in many tumors of epithelial origin [25].

To better understand the involvement of c-Myb in hematopoietic development, many efforts have been recently made to identify novel genes directly targeted by this transcription factor [26–29]. A recent study based on global gene expression profiling of *c-myb* siRNA-silenced erythroleukemic K562 cells identified novel target genes (*MYADM*, *LMO2*, *STAT5A*, *GATA2*, and *IKZF1*), which were further confirmed by chromatin immunoprecipitation (ChIP) assays [30]. Among them, three were recognized as transcription factors. This study suggested that *c-myb* may act as a key master regulator of gene expression, but can also coordinate gene activity within a more complex network including other transcription factors. A comparison of results from different profiling surveys, focusing on c-Myb transcription regulation, demonstrated that some discrepancies among the identified target genes may exist [26–29]. These differences were associated with the nature of the hematopoietic cells used in these studies.

The gene expression profiling investigations mentioned were all performed at transcriptome level. Although accurate and exhaustive, these studies do not go further into the complexity of the regulatory networks taking place within the cells, including the post-translational and degradative mechanisms. In this work, we originally describe the use of a differential proteomic approach to facilitate the comprehension of the regulation of the protein networks exerted by c-Myb. To this purpose, *c-myb* RNAi-silenced K562 cells and the corresponding parental K562 cell line were comparatively analyzed on a time-course basis by two Dimensional–Differential In Gel Electrophoresis (2D-DIGE). Differentially-represented spots were further identified by nLC-ESI-LIT-MS/MS, providing novel actors targeted by this transcription factor and suggesting potential protein biomarkers useful to monitoring CML progression.

2. Material and methods

2.1. *shRNA transduction*

Inhibition of c-Myb was obtained by stable transduction of the doxycycline (Dox)-inducible lentiviral vector pLVsh-c-Myb in chronic myeloid leukemia K562 cells [31]. This vector encodes a shRNA directed against human *c-myb*, whose expression can be induced by sub-micromolar/micromolar concentrations of doxycycline. Lentiviral infection was performed as previously described [24].