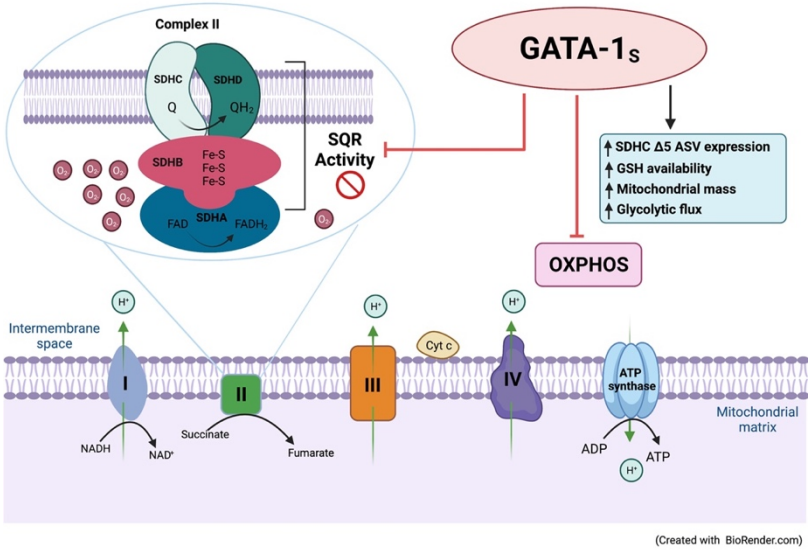
UNIVERSITY OF NAPLES FEDERICO II

DOCTORATE IN
MOLECULAR MEDICINE AND MEDICAL BIOTECHNOLOGY

XXXIV CYCLE

Silvia Trombetti

Exploring the leukemogenic potential of GATA-1$_S$, the shorter isoform of the hematopoietic transcriptional factor GATA-1, through mechanisms of mitochondrial remodeling, metabolic rewiring and apoptosis resistance associated with modulation of the cellular redox state



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Exploring the leukemogenic potential of GATA-1_S, the shorter isoform of the hematopoietic transcriptional factor GATA-1, through mechanisms of mitochondrial remodeling, metabolic rewiring and apoptosis resistance associated with modulation of the cellular redox state

Tutor
Prof. Michela Grosso

Candidate
Dr. Silvia Trombetti

2018-2022

Summary

LIST OF ABBREVIATIONS

Abbreviation	Meaning
AMKL	Acute megakaryoblastic leukemia
AML	Acute Myeloid Leukemia
ATP	Adenosine triphosphate
CCND2	Cyclin-D2
COX	Cyclo-oxigenase
C-TAD	C-terminal transactivation domain
CYP	Cytochrome P 450 monooxygenase
ECAR	Extracellular acidification rate
EBPα	Enhancer-binding protein α
ETC	Electron transport chain
FAD	Flavin adenine dincleotide
FBS	Fetal bovine serum
FCCP	Trifluoromethoxyphenylhydrazone
FOG	Friend of GATA
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Glutathione
GST	Glutathione S-transferase
HIF1	Hypoxia Inducible Factor 1
HSCS	Hematopoietic Stem Cells
KLF1	Kruppel Like Factor 1
MEP	Megakaryocyte/erythroid progenitors
MK	Immature megakaryocytes
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
NO	Nitric oxide
N-TAD	N-terminal activation domain
OCR	Oxygen consumption rate
OXPHOS	Oxidative phosphorylation system
PBS	Phosphate buffered saline
PHD	Prolyl hydroxylase domain
RET	Reverse electron transfer
ROS	Reactive oxigen species

SDH	Succinate dehydrogenase
SDHA	Subunit A of succinate dehydrogenase
SDHB	Subunit B of succinate dehydrogenase
SDHC	Subunit C of succinate dehydrogenase
SDHD	Subunit D of succinate dehydrogenase
SOD	Superoxide dismutase
SRC	Spare respiratory capacity
TAM	Transient myeloid leukemia
TF	Transcription factor
XLTT	X-linked thalassemia with thrombocytopenia
XOR	Xanthine oxidoreductase
TPA	Tissue plasminogen activator

ABSTRACT

Maintenance of a balanced expression of the two isoforms of the transcription factor GATA-1, the full-length protein (GATA-1_{FL}) and its shorter isoform (GATA-1_S), contributes to control hematopoiesis, whereas their dysregulation can alter the differentiation/proliferation potential of hematopoietic precursors thereby eventually leading to a variety of hematopoietic disorders. Although it is well established that these isoforms play opposite roles in these remarkable processes, most of the molecular pathways involved remain unknown. This thesis demonstrates that GATA-1_{FL} and GATA-1_S differentially influence mitochondrial remodeling, intracellular redox state and reactive oxygen species (ROS) compartmentation in K562 cell line and that the resistance to apoptosis in GATA-1_S cells is directly related to enhanced antioxidant capacity associated with GATA-1_S abnormal expression. GATA-1_S over-expression has also been found to be associated with high levels of the succinate dehydrogenase subunit C (SDHC). Based on the evidence that SDHC is over-expressed in several tumors with alternative splicing variants acting as potent dominant negative inhibitors of Succinate dehydrogenase (SDH activity), the levels of SDHC variants and the rate of oxidative mitochondrial metabolism have been examined in K562 cells over-expressing GATA-1 isoforms. Over-expression of SDHC variants accompanied by decreased SDH complex II activity and oxidative phosphorylation (OXPHOS) efficiency was found associated only with GATA-1_S over-expression. Given the tumor suppressor role of SDH and the effects of OXPHOS limitations in leukemogenesis, identification of a link between GATA-1_S and impaired complex II activity unveils novel pro-leukemic mechanisms triggered by GATA-1_S. Abnormal levels of GATA-1_S and SDHC variants were also found in an acute myeloid leukemia (AML) patient, thus supporting *in vitro* results. A better understanding of these mechanisms can contribute to identify novel promising therapeutic targets in myeloid leukemia.

1. INTRODUCTION

1.1 The GATA transcription factor family

The GATA family of transcription factors consists of six proteins (GATA1-6) so called after their common DNA binding consensus sequence (A/T) GATA (A/G) that is characterized by two C4 (Cys-X2-Cys-X17-Cys-X2-Cys) zinc-finger motifs that, along with an adjacent conserved highly basic region, constitute the DNA binding domain. The DNA binding regions are highly homologous within the GATA family members (more than 70% similar sequences). GATA factors are involved in a variety of physiological and pathological processes. They have been shown to play critical roles in development, cell-fate specification, regulation of differentiation and control of cell proliferation and movement. During vertebrate development, GATA transcription factors play pleiotropic roles in the early stages of cell differentiation and organ development in a variety of tissues (Tremblay, Sanchez-Ferras, and Bouchard 2018).
The GATA family can be divided into two subfamilies: GATA-1/2/3 are required for differentiation of mesoderm and ectoderm-derived tissues, including the hematopoietic and central nervous system; GATA-4/5/6 are implicated in development and differentiation of endoderm- and mesoderm-derived tissues and in the induction of differentiation of embryonic stem cells, cardiovascular embryogenesis and guidance of epithelial cell differentiation in the adult (Lentjes et al. 2016).

1.2 GATA transcription factor modes of action

The classical function of transcription factors is to bind specific DNA sequences within enhancer and promoter regions and to modulate their transcriptional output. However, a subclass of transcription factors called ‘pioneer transcription factors’ has the capacity to recognize and bind heterochromatic DNA sequences and promote chromatin opening and recruitment of additional transcriptional regulators, thus contributing to chromatin remodeling. In recent years, several examples of pioneer activity have been reported for GATA transcription factors. Through their pioneer activity, GATA proteins act as primary regulators of lineage decisions and cell fate transitions (Fig. 1A).
The pioneer activity of GATA factors and their subsequent role as classical transcriptional regulators are achieved largely via their interaction with co-regulators to assemble transcriptional complexes and recruit chromatin remodeling proteins (Tremblay, Sanchez-Ferras, and Bouchard 2018). Indeed, as part of their function in transcriptional regulation, GATA factors can contribute to 3-dimensional chromatin reorganization. Together with FOG, LDB1, MED1 and BRG1, GATA factors can promote chromatin looping by

bringing together distant enhancers and promoter elements (Fig. 1B) (Vakoc et al. 2005). As an example, a direct role in bridging distant regulatory elements has been demonstrated for GATA-1 at the β-globin locus. The differential recruitment of GATA factors and their regulatory complexes can also be achieved by changes in their expression pattern. They can influence each other's expression so that they can function consecutively during lineage commitment. This sequential activity of GATA factors on their target genes is referred to as the 'GATA switch' (Fig. 1C).
Furthermore, the identification of GATA cofactors and their associated complexes was used to reveal a transcriptional activity played by GATA factors. GATA factors can act as pioneers, activators and transcriptional repressors depending on the associated molecular complexes. Therefore, these multiprotein complexes can function as both activators and repressors of target genes (Fig. 1D-E).Alternatively, GATA factors can also antagonize each other for mutual co-factors interactions (Fig. 1F) (Tremblay, Sanchez-Ferras, and Bouchard 2018).

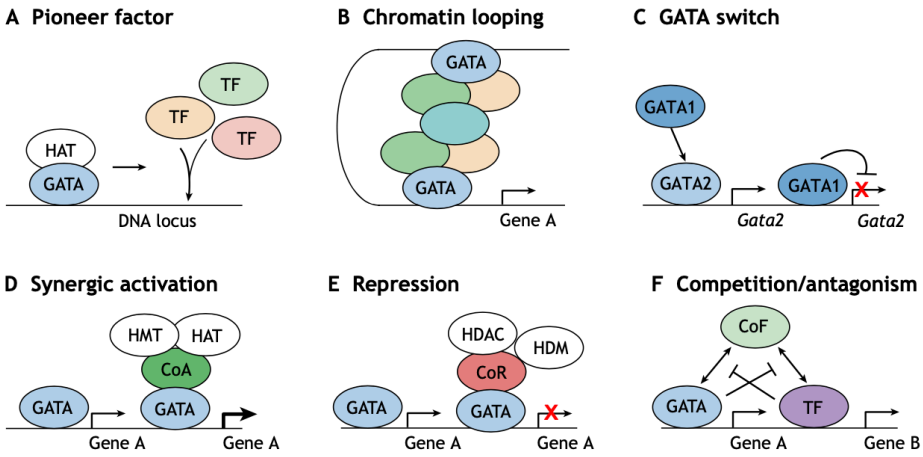


Figure 1: *Proposed modes of action of GATA transcription factors. A) GATA proteins can act as pioneer factors. B) GATA proteins participate in chromatin looping. C) Mechanism of GATA switch. D) GATA factors can also synergize with a co-activator to induce gene activation through recruitment of a histone methyl transferase (HMT) and/or histone acetyl transferase (HAT). E) Association of GATA factors with a co-repressor that can recruit histone demethylase (HDM) and/or histone deacetylase (HDAC) activities, negatively regulates gene expression. F) Finally, GATA factors can antagonize each other by competing for a mutual co-factor (from Tremblay, Sanchez-Ferras, e Bouchard 2018).*

1.3 GATA-1 and target genes

GATA-1 (also known as NF-E1, NF-1, Eryf-1 and GF-1), the founding member of the GATA family, was firstly identified as a protein that binds two adjacent sites within a 3' enhancer region of the chicken α-globin locus. The enhancer-binding activity was observed to be confined to extracts from erythroid cells,

suggesting that GATA-1 may be a regulatory protein with an important role in erythroid development. Subsequently, studies in mice and isolated human cells highlighted that GATA-1 stimulates the expression of genes that promote the differentiation in erythroblasts and down-regulates while silencing genes that promote cell proliferation. However, it is also expressed in megakaryocytes, eosinophils, and mast cells (Harigae 2006; Ling and Crispino 2020).
The human GATA-1 gene, encompassing a 7.74 kb region, is located on the short arm of the X chromosome at position 11.23. GATA-1 is composed of an untranslated first exon, the hematopoietic-specific first exon (IE) and five coding exons (Fig. 2). Interestingly, mouse and rat GATA-1 genes contain an additional testis-specific first exon (IT) upstream of the main first exon IE. In rodent animals the GATA-1 transcription unit is composed of two alternative untranslated first exons, IT and IE, and five translated exons, from II to VI. Exon IT is primarily used in testis Sertoli cells and it is located 8 kb upstream of exon IE, while exon IE is hematopoietic-specific. GATA-1 proteins expressed in hematopoietic and Sertoli cells are identical since exon II harbors the translation start site. The two zinc-finger motifs are encoded by exons IV and V, respectively (Ling and Crispino 2020; Ferreira et al. 2005; Kobayashi and Yamamoto 2007).

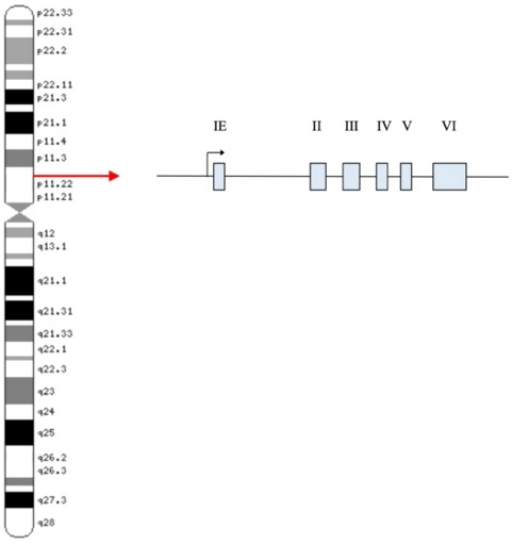


Figure 2: *The human GATA-1 gene locus on the short arm of the X chromosome at position Xp11.23*

Four functional domains have been identified within the GATA-1 protein (Fig. 3): an N-terminal acidic activation domain (N-TAD), an N-terminal zinc finger (N-finger), a C-terminal zinc finger (C-finger) and a C-terminal activation domain. The C-finger domain is critical for GATA-1 function, since it is responsible for the recognition of the GATA consensus sequence and for the consequent binding to DNA (Fig. 4) whereas the N-finger contributes to the stabilization and specificity of the DNA binding and mediates the formation of

complexes with other cofactors such as FOG-1 (**F**riend **O**f **G**ATA-1). A study using reporter assays in non-erythroid cells showed that the first N-terminal 80 amino acids are critical for GATA-1 transcriptional activity. This transactivation domain is also required for GATA-1-mediated terminal erythroid differentiation and megakaryocytic cell differentiation. The acidic domain can form an amphipathic α-helical structure and interacts with TFIID, stabilizing the general transcription apparatus and promoting transcription initiation (Ling and Crispino 2020). Although N-TAD is known to act as a solitary transactivation domain for GATA-1 clinical observations in Down syndrome (DS) patients with leukemia suggest that there may be additional transactivation domains. Deletion of the C-terminal region is associated with reduced transactivation activity of GATA-1 without significant attenuation of the DNA binding activity or self-association potential. Thus, hematopoietic GATA factors appear to harbor two independent transactivation domains, the acidic domain in the N-terminal region and a proline-rich domain in the C-terminal region (C-TAD) (Fig. 3). Both N-TAD and C-TAD retain redundant as well as specific activities for proper hematopoiesis *in vivo* (Kaneko et al. 2012).

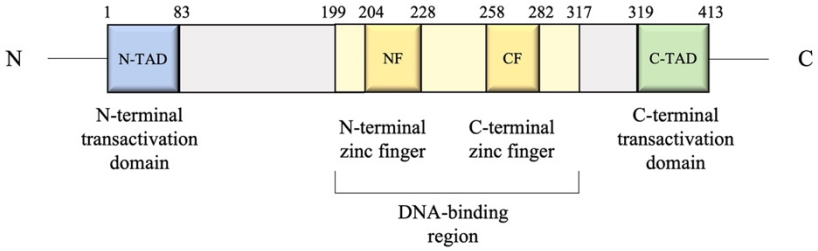


Figure 3: *GATA-1 protein domains. Four functional domains have been identified within the GATA-1 protein: the N-terminal acidic activation domain, the N-terminal zinc finger (N- finger), the C-terminal zinc finger (C-finger) and the C-terminal proline-rich activation domain.*

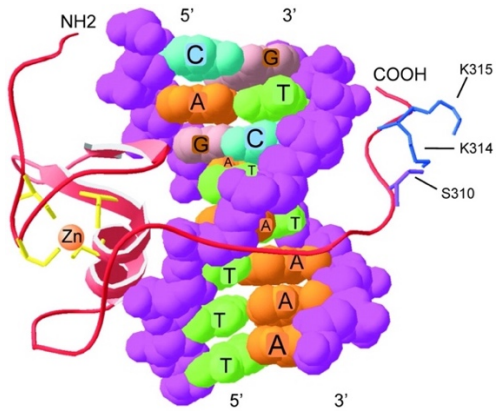


Figure 4: *Three-dimensional representation of GATA-1 C-finger domain bound to DNA. The C-finger domain is critical for GATA-1 function, since it is responsible for recognition of the GATA consensus sequence and DNA binding (from Ferreira et al. 2005).*

1.4 GATA-1 in hematopoiesis: co-factors and target genes

GATA-1 is expressed in primitive and terminally differentiated erythroid cells, megakaryocytes, eosinophils, and mast cells (Ferreira et al. 2005). From earlier progenitors toward the terminally mature cells, GATA-1 expression levels fluctuate. Upon hematopoietic differentiation, lineage-specific gene expression programs are controlled by GATA-1 to generate the diversity of cellular function in each hematopoietic lineage (Moriguchi and Yamamoto 2014) (Fig. 5).
GATA-1, the master transcription factor of erythropoiesis, transcriptionally regulates all processes related to erythroid maturation and function. GATA-1 gene knock-out in mice results in embryonic lethality due to severe anemia, with GATA-1-null cells undergoing apoptosis at the proerythroblastic stage. The conditional erythroid knock-out in adult mice causes aplastic anemia, revealing its essential role in both steady-state and stress erythropoiesis (Gutiérrez et al. 2020). Genome-wide occupancy studies (ChIPseq) have confirmed that all erythroid genes including EpoR (erythropoietin receptor), GATA-2, NF-E1, KLF1 (Kruppel Like Factor 1) are regulated by GATA-1.
GATA-1 transcription functions in erythropoiesis are mediated by the formation of activating and repressive transcription complexes, through GATA-1 interactions with several other transcription factors, cofactors, and chromatin remodeling and modification proteins.
GATA-1 and the myeloid transcription factor PU.1 have been reported to physically interact in antagonizing each other's functions (Stopka et al. 2005). The N-terminal activation region (N-TAD) of PU.1 interacts with the conserved C-Finger of GATA-1. PU.1 represses GATA-1-mediated transcriptional activation by blocking GATA-1 binding to DNA, thus inhibiting erythroid differentiation (Morceau et al. 2004). Many studies found that the N-TAD of PU.1 binds the C-pocket of the retinoblastoma protein (pRb) to repress GATA-1 activity through histone H3K9 methylation. Furthermore, GATA-1 inhibits PU.1 function by displacing the PU.1 coactivator c-Jun and blocking transcription by inhibiting histone H3K9 (P Burda, Laslo, and Stopka 2010; Pavel Burda et al. 2016). More recent data indicate that GATA-1 can bind PU.1 gene at the URE thus mediating its repression in human AML erythroleukemic blasts trough a mechanism that include repressive epigenetic remodeling of the URE region. Interestingly this important mechanism for PU.1 downregulation during progenitor cell differentiation could be employed during leukemogenesis (Pavel Burda et al. 2015).
GATA-1 also plays a critical role in megakaryocytic development. GATA-1-deficient mice were shown to have reduced platelet counts as well as an expansion of immature dysmorphic megakaryocytes exhibiting marked hyperproliferation *in vivo* and *in vitro* (Muntean and Crispino 2005). At the

molecular level, GATA-1 activates transcription of megakaryocyte specific genes including NF-E2, GP1bα (glycoprotein Ib α chain), PF4 (platelet factor 4) and Syk (Muntean and Crispino 2005).
Cooperation between FOG-1 and GATA-1 has been demonstrated during both erythroid and megakaryocytic cell differentiation since they synergistically activate the transcription of hematopoietic-specific genes. In addition, FOG-1 has been shown to repress eosinophil-specific gene expression through GATA-1 bound to eosinophil-specific promoters, and to block eosinophil differentiation. A similar role for FOG-1 in suppression of mast cell gene expression and differentiation has been demonstrated. These observations indicate that FOG-1 plays a key role in hematopoietic lineage bifurcation (Mancini et al. 2012). Insights into how FOG-1 performs activating and repressive functions derived from the identification of interacting proteins, among which the nucleosome remodeling and histone deacetylase complex NuRD, that binds to a small conserved motif at the extreme N-terminus of FOG-1 (Gregory et al. 2010).
It is well established that myeloid progenitor expression of the TF GATA-1 is essential for eosinophil lineage commitment. It was firstly shown that GATA-1 could convert chicken myeloblasts, mouse common lymphoid progenitors and human myeloid progenitors to eosinophils. Disruption of GATA sites within the GATA-1 promoter resulted in selective loss of the eosinophil lineage. More recently these findings were supported by global gene expression profiling analysis of single murine multipotent progenitor cells revealing that the eosinophil lineage commitment segregated with GATA-1 expression. At a molecular level, GATA-1 has been shown to activate expression of eosinophil specific genes such as MBP (Fulkerson 2017; Du et al. 2002). Besides expressing GATA-1, eosinophils express relatively high levels of the transcriptional factor C/EBPα (CCAAT/enhancer-binding protein α) to promote their terminal differentiation (Fulkerson 2017).
Mast cell development was also found to be disrupted in mice knocked-down for GATA-1 with mast cells showing defective terminal maturation and increased apoptosis rate (Migliaccio et al. 2003; Masuda et al. 2007). However, Ohneda et al. subsequently observed that GATA-1 knock-out has a minimal effect on the number and distribution of peripheral tissue mast cells in adult mice. This result led to hypothesize that GATA-1 is dispensable for mast cell differentiation and that it might play other specific roles in mast cell functions (Ohneda et al. 2014).

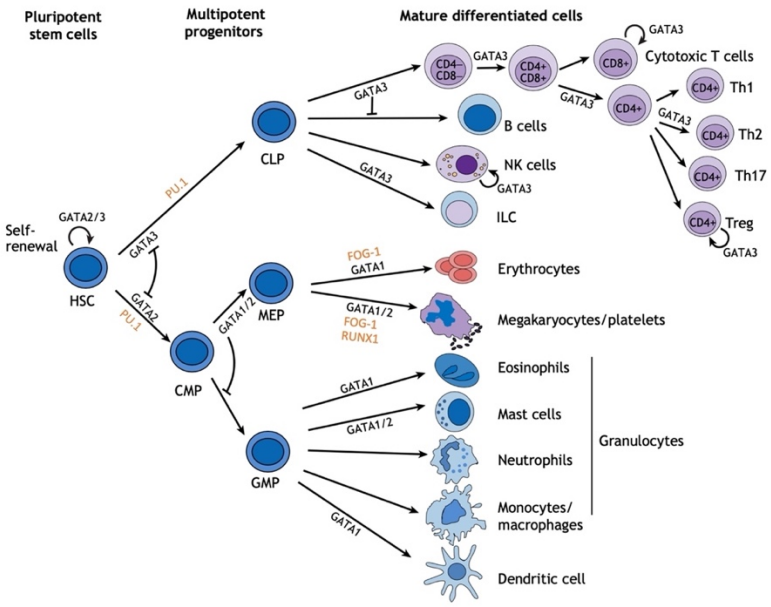


Figure 5: *Schematic representation of the main lineage commitment steps during hematopoiesis. Hematopoietic GATA factors and GATA-1 cofactors (shown in orange) required for the development of specific hematopoietic lineages are shown. ((from Tremblay, Sanchez-Ferras, and Bouchard 2018)*

1.5 GATA-1 isoforms, synthesis mechanisms and differential roles in hematopoiesis

GATA-1, a master regulatory transcription factor of key hematopoietic genes in several myeloid cell types, is physiologically present as two protein isoforms that originate from alternative splicing. (Fig. 6) (Halsey et al. 2012). The full-length GATA-1 protein (GATA-1_{FL}) comprises 413 amino acids with a predicted molecular weight of 48 kDa. The short isoform of GATA-1 (GATA-1_S), with a predicted molecular weight of 38 kDa, lacks exon two and uses an alternative translation initiation site (Met84). This isoforms lacks the first 83 amino acid residues corresponding to the N-terminal activation domain (N-TAD) (Gruber and Downing 2015). Both proteins contain two Zinc finger domains mediating DNA binding and protein interactions. Relative expression levels of GATA-1 isoforms are thought to be important for appropriate erythroid-megakaryocytic development.

A balanced GATA-1_{FL}/GATA-1_S ratio plays an important role in determining cell fate during hematopoiesis with the short isoform mainly involved in the maintenance of the proliferative potency of hematopoietic precursors whereas, conversely, GATA-1_{FL} is required to promote the terminal differentiation of erythroid-megakaryocytic lineages through the orchestration of sophisticated transcriptional networks (Chlon et al. 2015; Halsey et al. 2012; Kaneko et al. 2012; Doré and Crispino 2011).

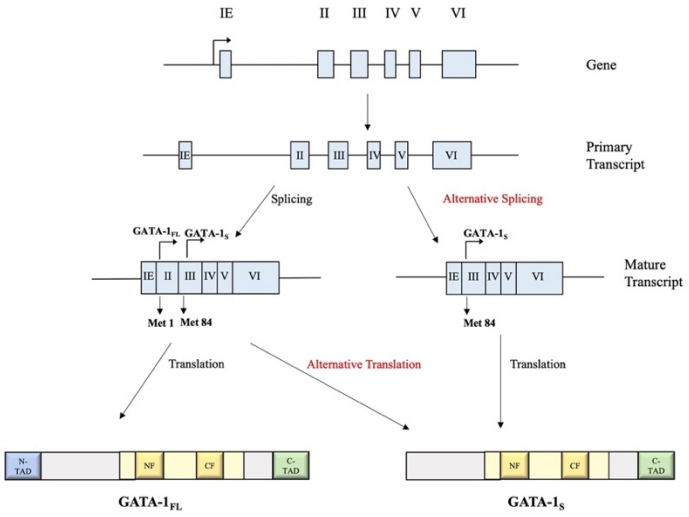


Figure 6: *GATA-1 alternative splicing processes.*

GATA-1_S efficiently binds DNA *in vitro* and interacts with FOG-1 and other cofactors similarly to the full-length isoform. However, due to the lack of the N-terminal activation domain, the shorter variant has reduced transactivation potential thus suggesting that GATA-1_S may act as a dominant-negative variant of the full-length protein. During normal hematopoiesis, when both GATA-1 isoforms are produced, GATA-1_S may down-modulate GATA-1_{FL} function (Gurbuxani, Vyas, and Crispino 2004). In order to characterize the transcriptional activity of GATA-1_S during the erythroid and megakaryocytic development, Chlon et al. (2015) performed a comprehensive analysis in order to understand how GATA-1_{FL} and GATA-1_S influenced gene expression and chromatin occupancy in G1ME cells showing both erythroid and megakaryocytic differentiation potential (Chlon et al. 2015).
Their data showed that GATA-1_S can induce megakaryocytic differentiation even though it binds and activates erythroid genes less efficiently than GATA-1_{FL}, thus revealing an impaired ability to properly promote development in erythroid cells. This study thus contributed to highlight GATA-1_S deficiency in erythroid specific gene expression activity (Chlon et al. 2015).
Halsey et al. obtained interesting data in K562 cells, a well-established *in vitro* model of hemin-induced erythroid differentiation. Whereas GATA-1_{FL} expression remained relatively stable during the differentiation process, GATA-1_S showed significant up-regulation at 6 h and down-regulation at day 3, as compared to baseline expression levels. When K562 cells were treated with TPA (Tissue Plasminogen Activator) to induce megakaryocytic differentiation GATA-1 isoforms initially showed reduced expression levels but thereafter GATA-1_{FL} expression increased whereas GATA-1_S transcripts remained at low levels. These results led to hypothesize that GATA-1 isoforms are independently

regulated at the RNA level during cell differentiation and that their expression changes according to specific molecular requirements. Halsey et al. also examined whether GATA-1_S and GATA-1_{FL} isoforms were associated with differential transcriptional profiles in K562 stably transfected with GATA-1_S or GATA-1_{FL} (Fig. 7) (Halsey et al. 2012).

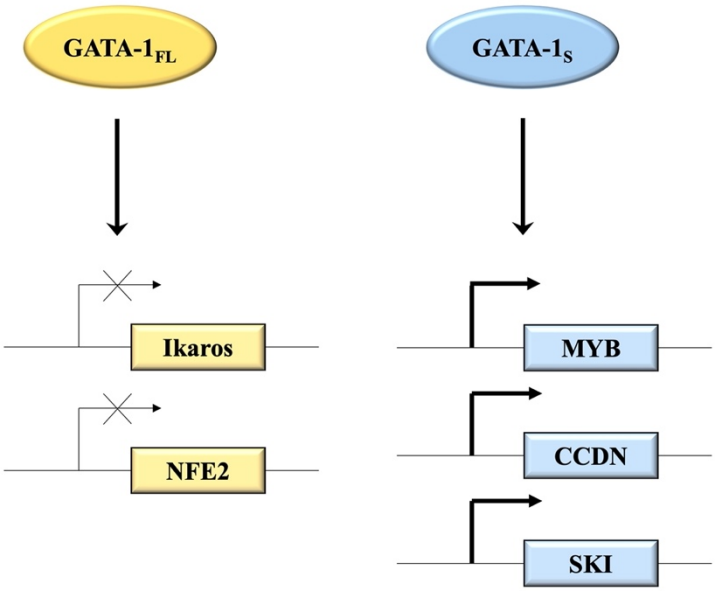


Figure 7: *Differential transcriptional profiles between GATA-1_{FL} and GATA-1_S.*

Their results showed that both Ikaros and NFE2 genes were found to be down-regulated in GATA-1_{FL} over-expressing cells with respect to GATA-1_S. Persistent expression of Ikaros is thought to be consistent with the maintenance of a primed multipotent progenitor state; its haploinsufficiency has been observed during the myeloproliferative transformation to acute myeloid leukemia (Malinge et al. 2013; Jäger et al. 2010). Moreover, impaired repression of NFE2, a key megakaryocytic transcription factor, may partially explain the strong association of GATA-1_S with acute megakaryoblastic leukemia (AMKL). Mice deficient in either GATA-1 or NF-E2 develop a phenotype characterized by a marked increase in abnormal, immature megakaryocytes (MKs) with thrombocytopenia (Kacena et al. 2013). Significant up-regulation of MYB, CCND2 and SKI was found concomitant with GATA-1_S over-expression. MYB is a key hematopoietic transcription factor involved in stem cell self-renewal. GATA-1_S promotes MYB expression at higher levels than GATA-1_{FL}. Even in this case, this finding is consistent with the involvement of GATA-1_S in the maintenance of a primitive progenitor state. Similar results were found for CCND2, a cell cycle regulator involved in megakaryocytic differentiation, whose down-regulation during terminal erythroid differentiation is triggered

only by GATA-1_{FL}. Finally, SKI, an important component of the histone deacetylase complex is over-expressed in acute myeloid leukemia. SKI is important because it shows an expression peak in murine megakaryocyte/erythroid progenitors (MEP) and it is able to block erythroid differentiation cells through direct interaction with the GATA-1 C-finger domain. These observations suggest that GATA- 1_S/SKI interaction may be important for the maintenance of an expanded MEP compartment with terminal differentiation blockage (Halsey et al. 2012). Another interesting finding regarding GATA-1 isoforms concerns their differential effect on the expression levels of WT1, a zinc finger transcriptional factor involved in cellular proliferation, differentiation and apoptosis. WT1 plays a role in hematopoiesis; it is up-regulated in immature leukemia cells and therefore it is a good clinical biomarker for disease progression, diagnosis and detection of minimal residual disease in myeloid leukemia (Rampal and Figueroa 2016). Interestingly, GATA-1_{FL} and GATA-1_S over-expression in K562 cells resulted in a more dramatic increase of WT1 expression exerted by GATA-1_S as compared to GATA-1_{FL} (Petruzziello et al. 2013). Complex transcription factor networks play a crucial role in the orchestration of myeloid cell fate and in the differentiation block observed in many myeloid leukemias (Paul et al. 2016; Rosmarin, Yang, and Resendes 2005). In this regard, dysregulated expression of GATA-1 and unbalanced GATA-1_{FL}/GATA-1_S expression are emerging as a key factor in malignant hematopoiesis such as transient myeloid leukemia (TAM) and acute megakaryoblastic leukemia (AMKL-DS) observed in children with Down syndrome (Bresnick et al. 2012; Gao, Chen, and Peterson 2015; Lentjes et al. 2016) commonly characterized by a prevalent expression of GATA-1_S with respect to its full-length isoform (Crispino 2005; Petruzziello et al. 2013). Also, elevated GATA-1_S levels have been recognized as a poor prognostic factor, thus further emphasizing the pro-leukemic role of this isoform in hematological malignancies (P Burda, Laslo, and Stopka 2010; Schulze and Italiano 2016; Ciovacco, Raskind, and Kacena 2008; Gao, Chen, and Peterson 2015; Shimamoto et al. 1995).

1.6 GATA-1 and apoptosis

Several lines of evidence support the theory that GATA-1 is directly involved in cell survival. GATA-1 activates the transcription of EpoR whose signaling is important for survival of erythroid progenitors. Also, Bcl-XL, a gene encoding an anti-apoptotic protein, is among the known target genes of GATA-1 (Suzuki et al. 2013).
Recent studies suggest that another possible GATA-1 function is the regulation of the G1/S cell cycle progression. Cell cycle control is of the utmost importance in hematopoietic differentiation, since progenitors must be able to proliferate in order to proceed through the hematopoietic development, even though terminal differentiation requires cell cycle exit. A variety of GATA-1 target genes are involved in cell cycle regulation or in proliferation and differentiation processes.

Moreover, absence of GATA-1 in megakaryocytes leads to increased proliferation and deficient maturation of megakaryocytic progenitors as well as to reduced number of circulating platelets. GATA-1 involvement in this mechanism was highlighted through the identification of missense mutations in the N-finger of GATA-1 found in patients with X-linked thrombocytopenia and anemia (Ferreira et al. 2005; Caldwell et al. 2013). Most of these mutations occur in the FOG-1 interaction surface of the N-finger, adversely affecting the binding of FOG-1 to the N-finger mutants. This piece of evidence further emphasizes the importance of the FOG-1/GATA-1 interaction. On the other hand R216Q, a mutation affecting the DNA face, shows lower affinity to palindromic but not to single GATA sites and is associated with XLTT (X-linked thalassemia with thrombocytopenia) (Harigae 2006; Balduini et al. 2004).
Furthermore, studies conducted on the CMK cell line (originated from blastic clones of acute megakaryoblastic leukemia isolated from a patient with Down syndrome) show that GATA-1_S expression alone gives these cells resistance to apoptosis through an increased expression level of the Bcl2 protein as compared to GATA-1_{FL} (Xavier et al. 2011).

1.7 Reactive oxygen species (ROS) in myeloid leukemia

Reactive oxygen species (ROS) are widely recognized as signaling molecules. ROS regulate a variety of cellular functions including proliferation, differentiation, epigenetic modification, and quiescence when equilibrium is ensured with detoxification (Trombetti, Cesaro, et al. 2021). The reactive oxygen species production causes damage to proteins, DNA, lipids and have effects on many cellular functions promoting tumor initiation, development and progression (Zorov, Juhaszova, and Sollott 2014; Lee et al. 2019).
Mitochondria are a primary source of ROS due to their unique role in aerobic metabolism and oxidative phosphorylation.
A controlled balance between ROS levels and antioxidant defenses is necessary for biological processes in both normal and tumor cells (Levine et al. 2017). In particular, AML is a very heterogeneous disease characterized by complex molecular and cytogenetic abnormalities (Kumar 2011), although some features including altered cellular redox status with high levels of ROS are common features of AML cells (Mattes, Vellenga, and Schepers 2019). The altered redox homeostasis affects the leukemogenic process of stem cells and hematopoietic progenitor cells that undergo mutational events correlated with an increased oxidative state. The result is an activation of oncogenes and consequent inactivation of tumor suppressor genes, increased aerobic metabolism and mitochondrial appearance (Zhang et al. 2015).
In particular, high ROS levels in AML cells are compensated by with antioxidant systems to avoid excessive ROS production and to protect leukemic cells from cell death induced by oxidative stress (Schieber and Chandel 2014; Snezhkina et al. 2019). However, given their high rate of ROS production, AML cells have a lower buffer capacity against ROS destruction than their normal counterpart.

This feature makes them more sensitive to pro-oxidant treatments than their normal counterparts (Kaweme et al. 2020).
Many altered metabolic pathways are described in acute myeloid leukemia such as xanthine oxidoreductase (XOR), uncoupled nitric oxide (NO) synthase (NOS), cytochrome P450 monooxygenase (CYP), cyclo-oxygenase (COX) and NADPH oxidase (Prieto-Bermejo et al. 2018; Pizzino et al. 2017; Moldogazieva et al. 2018). The NOX family is the first enzyme system reported to produce ROS as a primary function and not just as a byproduct of cellular metabolism.
Mitochondrial ROS takes place at complex I and complex III of electron transport chain (ETC) following a dispersion of electrons that are unable to reach complex IV and react with oxygen to form superoxide (O_2^-) (Fig. 8) (Brand 2016). Under mild oxidative stress conditions, defective mitochondria undergo mitophagy to reduce ROS generation and sustain cell survival whereas, on the contrary, high ROS levels conditions promote mitochondrial fission and dysfunction associated with perturbations in mitochondrial dynamics as so far reported for several pathologies including tumor initiation and progression (Snezhkina et al. 2019; Ježek, Cooper, and Strich 2018).Indeed, leukemia cells have respiratory failure and are more vulnerable to oxidative stress with respect to hematopoietic stem cells (HSCs) due to the increased mitochondrial mass and reduced respiratory activity (Al Ageeli 2020; Jitschin et al. 2014).

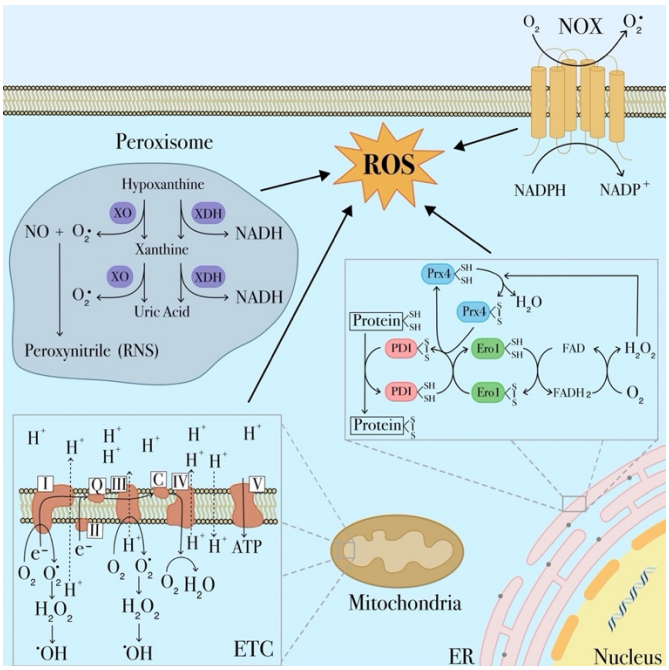


Figure 8. *Major sites of reactive oxygen species (ROS) production in leukemia cells. ROS are derived from different cellular compartments and enzymatic systems. The most significant source of ROS in the cell is represented by mitochondria, in which ROS are largely generated by the*

electron transport chain (ETC). Other ROS-producing mechanisms involve transmembrane NADPH oxidases (NOX), xanthine oxidoreductase in peroxisomes and protein disulfide isomerase (PDI) in endoplasmic reticulum (ER) (from Trombetti, Cesaro, et al. 2021).

1.8 Complexes of the mitochondrial respiratory chain and carcinogenesis

The mammalian mitochondrial electron transport chain (ETC) is a protein system that includes several electron carriers (complexes).
The ETC is composed of transmembrane protein complexes (I-IV) and of the freely mobile electron carriers ubiquinone and cytochrome c and it is located in the inner membrane of these organelles. Complexes I-IV along with F_1F_0ATP synthase are required for adenosine triphosphate (ATP) production during oxidative phosphorylation (OXPHOS) (Iwata et al. 1998; Guo et al. 2017) (Fig. 9). Substrates used by the electron transport pathway are NADH and FADH2. The electron flow is coupled to the generation of a proton gradient across the inner membrane and the energy accumulated in the proton gradient is used by complex V (ATP synthase) to produce ATP (Zhao et al. 2019).
Complex I (CI), also called NADH-ubiquinone oxidoreductase, is the largest multi-subunit enzyme complex in the ETC. The key role of this complex is to transfer electrons from matrix NADH to ubiquinone (Zhao et al. 2019).
Complex II (CII), namely succinate dehydrogenase (SDH), is the bridge enzyme between the Krebs cycle and the electron transport chain. SDH thus serves as a link between metabolism and OXPHOS (Cecchini 2003; Sun et al. 2005).
Complex III (*CIII)* (or ubiquinol-cytochrome c oxidoreductase) transfers the electrons received by UbQH2 from complex I and complex II to cytochrome c and couples this reaction with proton translocation across the inner mitochondrial membrane.
Complex IV (CIV), also known as cytochrome c oxidase, transfers electrons from cytochrome c to the terminal electron acceptor O_2 to generate H_2O.
Complex V (CV) or F1F0 ATP synthase, is a multi-subunit complex and consists of two functional domains: F0 and F1 connected by two stalks. F0 consists of 10 subunits, is embedded in the membrane and functions as a proton channel. F1 is the catalytic domain that uses the electrochemical gradient generated by the mitochondrial respiratory chain to convert ADP into ATP, the final step of the oxidative phosphorylation (Zhao et al. 2019; Jonckheere et al. 2013).
Recent studies demonstrated that many tumors exhibiting mutations in ETC components show a strong propensity to produce ROS, supporting the crucial role of this mechanism in modulating the phenotype of cancer cells (Raimondi, Ciccarese, and Ciminale 2020). In particular, complexes I and III have been identified as the most relevant ROS production sites within ETC in tumorigenesis and metastasis in several cancers (Raimondi, Ciccarese, and Ciminale 2020).
Mutations in complex I components can lead to increased O_2^- production, thus supporting ROS-dependent oncogenic pathways and inducing damage to

mitochondrial DNA. Besides influencing super-complex assembly, defects in the ND2 subunit are known to promote tumorigenesis and metastasis in breast, pancreatic and oral cancers. Similar phenotypes are related to mutations at the ND4 subunit, as found in AML, which are associated with increased ROS generation (Raimondi, Ciccarese, and Ciminale 2020). To demonstrate the importance of complex I in cancer progression, recent studies with metformin, a drug that acts as an inhibitor of complex I, resulted in a reduction in tumorigenesis both *in vitro* and *in vivo* (Wheaton et al. 2014). Under hypoxic conditions, NADH depletion and super complex disassembly hinder complex activity with ROS production mainly originating in this case from complex III (Bell et al. 2007). However, during hypoxia, complex I can produce ROS by reverse electron transfer (RET) (Raimondi, Ciccarese, and Ciminale 2020).
In cancer progression, the UQCR2 protein, an important subunit of the complex III has been shown to be up regulated in many human cancers, including lung adenocarcinoma, breast cancer and colorectal cancer. It is interesting to note that UQCR2 negatively regulates p53 levels by inducing its degradation, so that its expression increase attenuates cell cycle arrest and senescence, thus promoting tumorigenesis.
Although not directly involved in ROS production, complex IV activity has an influence on the electron flow, impacting electron loss from previous complexes, and cooperates with oncogenes, such as BCL-2, to support tumorigenesis. Mutations in subunits I and II of complex IV have been reported in several tumor types including ovary and prostate cancers and in AML. Interestingly, complex IV expression is stimulated by p53 and the frequent co-mutation of complex IV and TP53 in patients with AML correlates with worse prognosis, possibly due to increased mitochondrial DNA damage and mitochondrial dysfunction (Raimondi, Ciccarese, and Ciminale 2020).

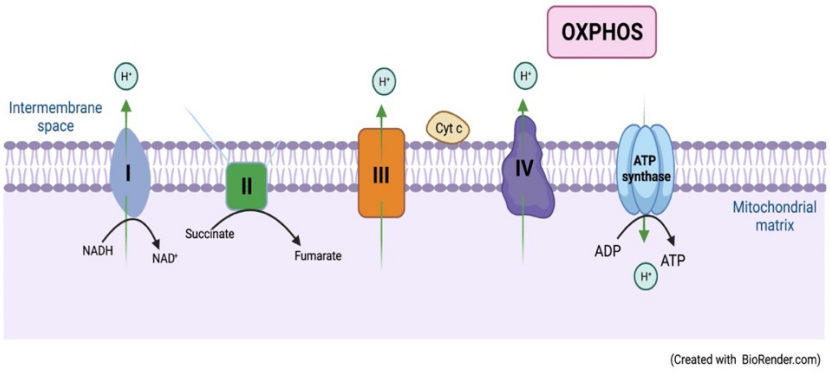


Figure 9: *Schematic representation of Mitochondrial electron transport chain (from Trombetti, Cesaro, et al. 2021).*

1.9 Succinate dehydrogenase (complex II) physiological function and deficiency

SDH complex, also designated as succinate-ubiquinone oxidoreductase or mitochondrial complex II, is the bridge enzyme between the Krebs cycle and the electron transport chain. SDH participates in the electron transfer and catalyzes the oxidation of succinate to fumarate with the reduction of ubiquinone to ubiquinol (Ciccarone, Di Leo, and Ciriolo 2018). This complex includes six protein subunits: SDHA, SDHB, SDHC, SDHD, SDHAF1, and SDHAF2 with the last two of them acting as associated accessory factors (Rutter, Winge, and Schiffman 2010). The SDHA and SDHB subunits containing the dehydrogenase catalytic constitute the hydrophilic head that protrudes into the matrix compartment while C and D subunits form the hydrophobic core anchored to the inner mitochondrial membrane and are also deputed to ubiquinone binding for ETC reactions (Lemarie and Grimm 2009; Dalla Pozza et al. 2020).
Flavin adenine dinucleotide (FAD) and Fe-S clusters, the two prosthetic groups of the catalytic core, participate to the electron transport from succinate to CoQ. Subunits C and D have some binding sites for the heme b560 moiety and two CoQ domains: the proximal high-affinity QP site and the distal low-affinity QD site. CoQ reduction occurs in two single-electron reactions, with the high-affinity QP site that markedly stabilizes the partially reduced semiquinone, thus allowing its complete reduction to $CoQH_2$ (Lemarie and Grimm 2009; Dalla Pozza et al. 2020).
The role of the heme fraction and the QD site remains to be clarified. Heme b is not required for CoQ reduction at the p site but is probably involved in the complex assembly in mammalian cells and acts as a mediator for electron transfer to the QD (low affinity) site. The heme b acts as an electronic pocket that inhibits the interactions between semiquinone CQ and O_2^- molecules that cause an uncontrolled ROS generation (Franco, Bremner, and Barros 2020; Bezawork-Geleta et al. 2017; Lemarie and Grimm 2009).
Besides a well-established function in mitochondrial metabolism, recently many studies have highlighted a role as tumor suppressor for SDH and as oncometabolite for its substrate succinate based on the evidence that SDH germline mutations have been found in several cancer types and contribute to abnormal intracellular and extracellular succinate accumulation (Brière et al. 2005; Dalla Pozza et al. 2020; Rutter, Winge, and Schiffman 2010).
Mutations in the SDH subunits leading to loss of function of complex II and consequent accumulation of succinate, increased ROS generation and decreased ATP production through OXPHOS have been found in various tumors such as hereditary pheochromocytoma, paraganglioma, renal cell carcinoma and gastrointestinal stromal tumors. However, in this context it is noteworthy that mutations occurring in different SDH genes lead to remarkable differences in clinical phenotype (Amar et al. 2021; Andrews et al. 2018).

Loss of SDH activity leads to accumulation of succinate that acts as a competitive inhibitor of enzymes belonging to the class of α-ketoglutarate-dependent dioxygenase including histone demethylases and prolyl 4-hydroxylase (PHD).
Defects in complex II activity can also lead to an increase in O_2^- production, a condition that is linked to several disease scenarios associated with oxidative stress such as cancer, leukemia and degenerative disorders. Several studies indicated that SDHC mutations result in increased O_2^- production, oxidative stress and genomic instability with consequent induction of apoptosis, thus contributing to critical features of the malignant phenotype (T. Ishii et al. 2005; Slane et al. 2006; N. Ishii, Ishii, and Hartman 2006; De Sousa et al. 2020)
Recent studies have shown that this process is coupled to cellular and mitochondrial acidification and, therefore, to lower mitochondrial pH values. Complex Environmental acidity conditions seem to influence the assembly of complex II subunits. In fact in these conditions the active SDHA / SDHB catalytic sub-complex dissociates from the SDHC / SDHB subunits anchored to the membrane with the Qp site being inaccessible (Sierotzki and Scalliet 2013; Lagadic-Gossmann, Huc, and Lecureur 2004)(Fig. 10).
Notably, this latter enzyme is involved in post-translational regulation of hypoxia inducible factor 1α (HIF-1α) stability. In fact, aberrant succinate levels can promote epigenetic alterations in cancer cells through succinate inhibition of PHD-mediated degradation of HIF-1α leading to constitutively activated HIF-1α survival pathway is constitutively activated leading to the transcription of genes that mediate the adaptive response to hypoxia, a common hallmark of cancer cells (Tretter, Patocs, and Chinopoulos 2016; Laukka et al. 2016; Cramer-Morales et al. 2021).

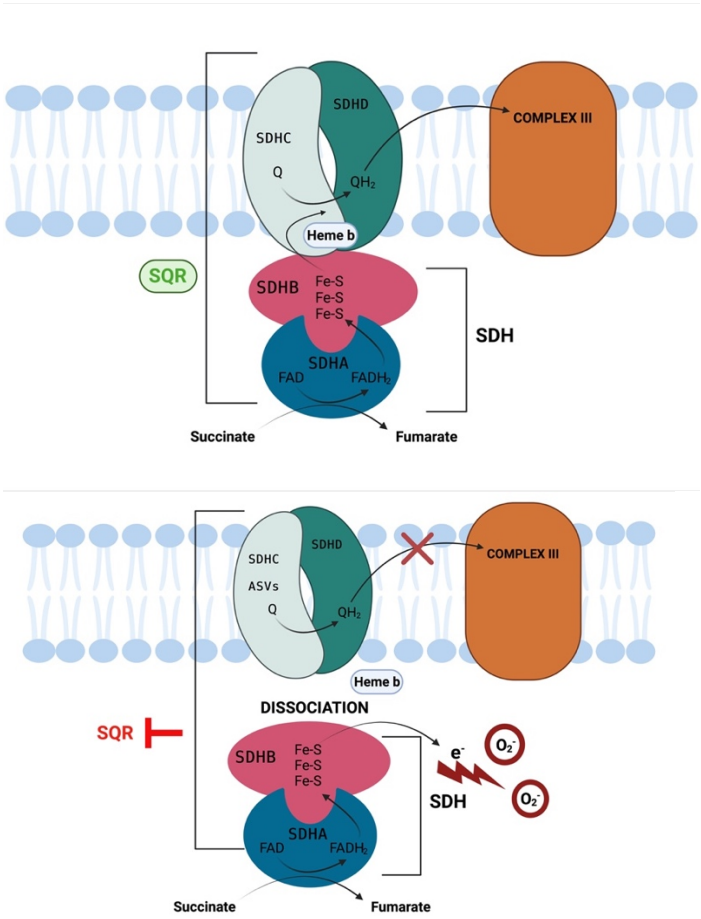


Figure 10: *Schematic representation of complex II disassembly induced by over-expression of the SDHC Δ5 variant lacking the heme binding site with impaired SQR activity and increased O_2^- production (from Trombetti, Cesaro, et al. 2021). (Created with BioRender.com, accessed on 6 August 2021).*

1.10 SDHC: isoforms and their role in ROS homeostasis

The human SDHC gene maps on the long arm of chromosome 1 and consists of six exons encoding for a 169-amino acid (aa) polypeptide corresponding to the full-length form of SDHC. Two main SDHC splicing variants have been described so far: the in-frame Δ3 ASV isoform lacking exon 3 with partial loss of the SDH oxidoreductase main activity region and the frameshift Δ5 ASV isoform, characterized by exon 5 skipping, loss of the heme binding domain and a 70-aa elongated C-terminal region (Fig. 11).

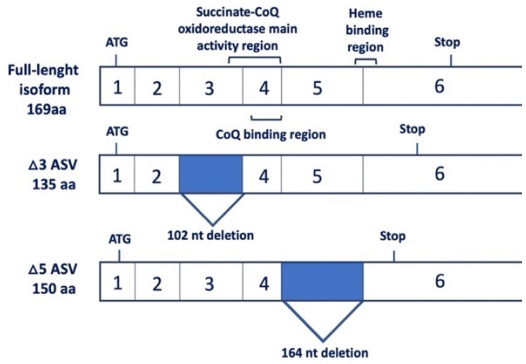


Figure 11: *Schematic representation of the alternative splicing mechanism generating SDHC variants (ASVs). Solid boxes and bars indicate the deleted exons and the corresponding protein domains, respectively.*

The Δ3 and Δ5 isoforms of SDHC have been associated with down-regulation of SQR activity with respect to the full-length isoform, supporting the theory that ASV isoforms act as negative dominant variants of the full-length protein (Satoh et al. 2015).
More recent studies have found that over-expression of isoforms Δ3 and Δ5 in HCT-15 colorectal adenocarcinoma cells is associated with reduced SDH activity. Furthermore, over-expression of the Δ5 isoform leads to an increase in the production of superoxide anion in HCT-15 cells compared to the levels generated by full-length SDHC. This altered oxidative stress can lead to over-expression of cancer-related factors (Satoh et al. 2015).

1.11 Antioxidant Defenses

Redox homeostasis is dependent on the balance between ROS production and antioxidant activities. Along with increased ROS production, defective antioxidant defenses in leukemia cells promote increased ROS levels and high oxidative stress status.
Indeed, abnormal antioxidant activities contribute to modulate ROS levels and maintain constitutive oxidative stress conditions without surpassing the threshold of an irreparable state of cell injury and thus avoiding activation of cell death pathways in different leukemia types (Snezhkina et al. 2019; Irwin, Rivera-Del Valle, and Chandra 2013; Er et al. 2007). Depending on different cell type scenarios variable antioxidant levels have been found. For example, superoxide dismutases (SOD) activity is increased or decreased in different types of leukemia: it appears to be reduced in ALL and increased in AML (Y. Wang et al. 2018; Battisti et al. 2008; Udensi and Tchounwou 2014; Nishiura et al. 1992).
SODs catalyze the dismutation of O_2^- to form H_2O_2 and O_2 and contribute to protect cells from oxidative stress. Three different isoforms of superoxide dismutase are known differing for ion cofactors and cellular localization:

• SOD1 (Zn-SOD), cytosolic with some expression in the mitochondrial inner membrane.
• SOD2 (Mn-SOD), mitochondrial
• SOD3, extracellular.

The presence of these compartment-specific isoforms indicates that SODs not only act as ROS scavenger and detoxifying enzymes but also play crucial roles in ROS homeostasis and ROS signaling between different cell compartments. (Holmström and Finkel 2014). However, evidence suggests that SOD expression is functionally required in AML and its inhibition leads to enhanced apoptosis in these cells (Huang et al. 2000; Chen and Kan 2015). Recent studies have shown that SOD2 over-expression in RGK1 cells of mouse gastrointestinal mucosa is related to decreased levels of superoxide anion. Furthermore, a significant decrease in the expression levels of GATA proteins, particularly GATA-1, was observed in these cells. This suggests that a decreased in mitochondrial superoxide anion content correlates with down-regulation of GATA expression levels and provide compelling evidence that mitochondrial ROS levels can influence cytosolic transduction signals.

The most abundant non-enzymatic ROS scavenger, glutathione (GSH) and its related enzymatic activities including glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) participate in H_2O_2 detoxification (Fig. 12) and play a critical role in different cellular processes such as proliferation, division, and differentiation (Kirtonia, Sethi, and Garg 2020; Ježek, Cooper, and Strich 2018). Alterations of the GSH metabolism have been observed in many cancer types, including hematological malignancies. Recently, Riccio et al. found that enhanced antioxidant capacities due to increased GSH levels and GSH/GSSG ratio in K562 cells, a human chronic myeloid leukemia cell line, were accompanied by over-expression of GATA- 1_S consistently with the pro-leukemic role associated to this specific protein isoform (Riccio et al. 2019).

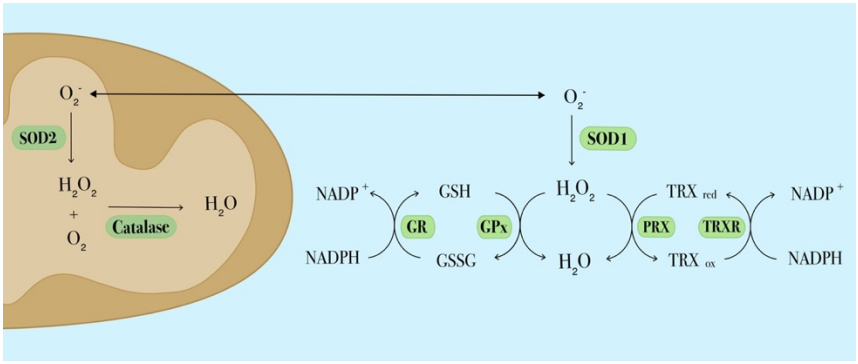


Figure 12: *Schematic diagram depicting the main antioxidant systems in leukemia cells. The superoxide dismutase (SOD) catalyzes the dismutation of superoxide into molecular oxygen and hydrogen peroxide, which is then further processed by catalase. Intracellular SOD isoforms have different locations: SOD1 is mainly located in the cytosol and also even at low level in the mitochondrial inner membrane whereas SOD2 shows a mitochondrial location. The glutathione*

(GSH) antioxidant system comprises GSH, glutathione reductase (GR) and glutathione peroxidase (GPx). To perform its antioxidant function, GSH needs to be oxidized into GSSG via GPx. To restore reduced GSH levels, GSSG is converted by GR in a reaction that requires NADPH. The thioredoxin (TRX) antioxidant system involves TRX, peroxiredoxin (PRX) and thioredoxin reductase (TRXR). Reduced TRX catalyzes the reduction of disulfides within PRX. In this process TRX is oxidized (TRXox) and subsequently reduced (TRXred) by thioredoxin reductase (TRXR) through a NADPH-dependent mechanism (from Trombetti, Cesaro, et al. 2021).

2. AIM OF THE STUDY

GATA-1 is a key hematopoietic transcription factor involved in myeloid differentiation since the early stages of hematopoiesis. The gene codes for at least two protein isoforms, namely GATA-1_{FL} and GATA-1_S, that are physiologically expressed in an adequate balance and exert different roles.
GATA-1_{FL} isoform reduces the expression level of several genes in order to promote differentiation and antiproliferative processes while GATA-1_S upregulates genes involved in cell proliferation and cell renewal. The aberrant over-expression of GATA-1_S leads to an unbalanced GATA-1_{FL}/GATA-1_S ratio that is described in several hematopoietic disorders including acute myeloid leukemia.
Based on these data and on the evidence that altered homeostasis of reactive oxygen species (ROS) is strongly involved in the development of hematological malignancies, my study was aimed to explore possible correlation between the expression of GATA-1 isoforms and different cellular redox states in leukemia cells.

Therefore, my aims were

- To verify whether cells overexpressing GATA-1_{FL} and GATA-1_S could differentially influence cell viability, apoptosis rate, the production of reactive oxygen species (ROS) and intracellular redox states in the K562 cell line;
- To verify if these variations could be dependent on differences in their antioxidant capacities;
- To verify if different redox states in these cells could be related to defects in electron transport chain, particularly at complex II (SDH) level;
- To evaluate possible correlations between the expression levels of GATA-1 isoforms and mitochondrial metabolism.

3. MATERIAL AND METHODS

3.1 Cell Culture

The human K562 cell line (ATCC, CCL-243), lymphoblasts isolated from the bone marrow of a 53-year-old chronic myelogenous leukemia patient, was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) plus 4 mM glutamine, 10 U/ml penicillin and 10 mg/ml streptomycin (all reagents from Gibco, Thermo Fisher Scientific Inc, Waltham, MA) at 37°C in a humidified 5% CO2-containing atmosphere. Cells were kept sub confluent for transient transfection experiments.

3.2 Transient transfection

K562 cells were transiently transfected with a mix containing 1 μg of p3XFLAG-GATA-1_{FL} (GATA-1_{FL} cells), p3XFLAG-GATA-1_S (GATA-1_S cells), or p3XFLAG-CMV empty vector (mock control) and 5 μL of Lipofectamine 2000 as transfection reagent (Invitrogen, Carlsbad, CA). Two hours before transfection, cells were plated into six-well plates at a density of 5 × 10^5 in 2 mL of Optimem medium (Invitrogen) without FBS. Five hours after transfection, the medium was supplemented with 10% FBS in each well. Forty-eight hours after transfection, cells were harvested for total RNA and protein extraction or were used for evaluation of oxidative stress and for 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) and Annexin V/propidium iodide (PI) assays.
For GATA-1_S knockdown experiments, K562 cells were seeded into a 24-well plate at a density of 2 × 10^5 in in 100 μL serum–free RPMI 1640 medium. A custom GATA-1_S small interfering RNA (GATA-1_S siRNA) was synthesized using the following target sequence: 5′-CCAGCCCAGTCTTTCAGGTG-3′ (Qiagen, GmbH, Hilden, Germany, #1027423) and transfected at final concentration of 50 and 100 nM. The required amount of GATA-1_S and mock control (Qiagen, #1027310) was diluted in 100 μL of serum-free Optimem (Invitrogen) and mixed with 6 μL of HiPerFect Transfection Reagent (Qiagen). The transfection mixtures were incubated for 15 min at room temperature and then added to the cells. Six hours after transfection, 400 μL of culture medium containing 10% FBS was added to each well. Forty-eight hours after siRNA transfection, K562 cells were collected for further analysis.

3.3 Assessment of cell viability and apoptosis

Cell viability was determined using the MTT assay. Briefly, after transient transfection, K562 cells were seeded into a 96-well plate at a concentration of 1.5 × 10^4 cells/100 μL. At 24, 48, and 72 hr after transfection, respectively, 10 μL of MTT labeling reagent (Cell Proliferation Kit 1, Roche, Mannheim,

Germany) was added to each well according to the procedures recommended by the manufacturer. Measurement of the soluble formazan product in each well was carried out by photometric reading at 570/690 nm on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT). Each transfection experiment was performed in triplicate.
Apoptosis resistance was assessed with an Annexin V-FITC Apoptosis Detection Kit 1 (BD Biosciences, San Diego, CA) according to the manufacturer's protocol. Forty-eight hours after transfection, cells were treated for 16 hr with two doses of cisplatin (10 and 20 μM) to induce apoptosis and were analyzed using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) and BD ACCURI C-Flow software.

3.4 Evaluation of ROS levels and compartmentation

Transiently transfected K562 cells were analyzed by flow cytometry and fluorescence microscopy for quantification of cytoplasmatic and mitochondrial ROS levels. Cytoplasmatic ROS were measured with CellROX deep red oxidative stress reagent (Thermo Fisher Scientific) a fluorogenic probe designed to reliably measure cytoplasmatic ROS in living cells. Forty-eight hours after transfection, cells were harvested, washed once with cold PBS, incubated in 5 μM CellROX reagent for 30min at 37°C, according to the manufacturer's protocol and analyzed by flow cytometry.
Cytoplasmatic ROS were also measured in wild-type K562 cells treated with 10 and 20 μM cisplatin, to verify if the pro-apoptotic effect of this drug could be mediated by an increase in ROS production. Treatment with menadione (Sigma-Aldrich, St. Louis, MO), a well known ROS inducer (Steinmeier and Dringen 2019) was used at 10 μM concentration for 16 hr as a positive control for ROS production.
Determination of superoxide ions production in mitochondria was performed using the MitoSOX red mitochondrial superoxide reagent (Thermo Fisher Scientific) that specifically target mitochondria where it is rapidly oxidized by superoxide anion but not by other reactive oxygen species. Transfected cells were harvested, washed once with cold PBS and were incubated with 5 μM MitoSOX reagent for 10 min at 37°C. Assessment of mitochondria mass was performed by incubating cells for 15 min in 100 nM MitoTracker Green FM reagent (Thermo Fisher Scientific) which stains mitochondria in a trans-membrane potential-independent manner and allows quantitative analysis by flow cytometry. After washing and resuspension in phosphate-buffered saline (PBS), all samples were analyzed using an Accuri C6 flow cytometer (Accuri C6) and BD ACCURI C-Flow software. Green (530/30 nm), orange (585/42 nm), and red (> 650 nm) fluorescence emissions were evaluated using logarithmic amplification. Data from 10,000 cells were collected, a sufficient number of events for statistical analysis of relatively small changes in relative fluorescence intensity (RFI). Mean RFI was determined after exclusion of debris and PI-positive events from the list mode data set. All samples were also

examined by fluorescence microscope analysis (DM14000; Leica Microsystems, Wetzlar, Germany).

3.5 Glutathione determination

Measurement of total (GSH+GSSG) or oxidized glutathione (GSSG) was accomplished with the GSH/GSSG-Glo Assay kit (Promega, Madison, WI). The GSH/GSSG ratio was calculated from net relative luminescence units according to the procedures recommended by the manufacturer. For each sample, analysis was performed in triplicate using 10,000 cells/well.

3.6 Quercetin treatment

Forty-eight hours after transfection, cells were treated with 50, 100, and 150 μM quercetin (Sigma-Aldrich) for 3 and 24 hr. After treatments, cells were collected and used for evaluation of glutathione levels and for cell viability and apoptosis assays. To perform cotreatments with quercetin and cisplatin, 48 hr after transfection, cells were treated with 50 or 150 μM quercetin for 8 hr and for additional 16 hr with 10 μM cisplatin.

3.7 Protein extraction

For protein extraction, K562 cells were collected 48 hr after transfection and washed twice with 4 mL of cold 1X PBS by centrifugation at 3,000g for 10 min at 4°C. Pellets were resuspended in 50 μL of lysis buffer (10% glycerol, 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% NP-40, 1 mM EDTA pH 8, 0.5 μL of protein inhibitor cocktail mixture (Sigma-Aldrich) and incubated for 30 min on ice. Samples were then centrifuged at 10,000 g for 30 min at 4°C and the supernatant containing the total protein extract was collected. Evaluation of protein concentration was performed by spectrophotometer analysis, with the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) according to the Bradford method. Protein extraction from bone marrow specimens from a patient with AML and from three healthy controls was performed using the Qiazol (Qiagen GmbH, Hilden, Germany) procedure according to the manufacturer's instructions. Informed consent for genetic studies was obtained from the investigated subjects in agreement with the Declaration of Helsinki.

3.8 Real-time PCR analysis

Total RNA was extracted from K562 cells with Qiazol reagent (Qiagen) according to the manufacturer's protocol. After spectrophotometric quantization, RNA quality was checked by gel electrophoresis on a 1.5% denaturing agarose gel in MOPS 1X buffer (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA pH 8.0). To quantitatively determine the SDHC expression levels, real-time PCR was performed using a CFX96 real-time system (Bio-Rad

Laboratories). cDNA was synthesized from 250 ng of total RNA using the QuantiTect Reverse Transcription Kit (Qiagen) and 2 µL of 7xgDNA wipeout buffer in a final volume of 14 µL to remove any traces of genomic DNA. The reaction was performed according to the kit protocol and subsequently used for quantitative real-time PCR procedures with the following primers (Table 1) of several genes. Glyceraldehyde-3-phosphate dehydrogenase GAPDH transcript was used as endogenous control.

Transcript	Accession number	Primer	Sequence 5′- 3′	Amplicon size
SDHC	NG_012767.1	For 1	CACTTCCGTCCAGACCGGA	100 bp
		Rev 1	CTGATACAGAGCTGAGGGCTAA	
SDHC ***full-length***	NM_003001.5	For 2	TCTGTATCAGAAATGCTGTTCC	183 bp
		Rev 2	GAGACCCCTGCACTCAAAGC	
SDHC ***Δ 3 ASV***	NM_001035512.2 (AB211234.1)	For 3	GCTCTGTATCAGAAATTGGTCT	250 bp
		Rev 3	GTCCCACATCAAGTGTCGGA	
SDHC ***Δ 5 ASV***	NM_001035511.2 (AB211235.1)	For 2	TCTGTATCAGAAATGCTGTTCC	187 bp
		Rev 4	GGTCCCACATCTGCACTCAA	
GAPDH	NM_002046.7	For	GAGCCACATCGCTCAGACAC	116 bp
		Rev	GGCAACAATATCCACTTTACCA	
HIF1 α	NM_001530.4	For	TCCAAGAAGCCCTAACGTGT	179 bp
		Rev	TGATCGTCTGGCTGCTGTAA	

Table 1. *Primer sequences used for quantitative real-time PCR analysis.*

Each real-time PCR was performed for triplicate measurements in a 20 µL reaction mix containing 10 µL of 2× SsoAdvanced Universal SYBR Green supermix (Bio-Rad Laboratories), 0.38 µL of a 20 µM primer mix, 2µL of cDNA (1/10 volume of RT-PCR product), and 7.62 µL of nuclease-free water.
The cycling conditions consisted of an initial denaturation step at 95°C for 3 min, followed by 40 cycles (95°C for 15 s, 60°C for 30 s) and 80 cycles performed according to standard protocols for melting curve analysis. The calibration curve for assessing the efficiency of the PCR reaction was performed on at least three serial dilutions (1:10) of the reverse transcriptase products. CT values were determined by automated threshold analysis and data were analyzed by the CFX Manager 3.0 software (Bio-Rad Laboratories) according to the manufacturer's specifications.
To evaluate the Real-Time PCR reaction, a calibration curve was performed using three serial dilutions (1:10; 1:100; 1:1000) of RNA samples isolated from peripheral blood and then reverse-transcribed in cDNA. The acceptance range of the threshold cycles (Cq) obtained for the housekeeping GAPDH gene was set between 20.0 and 30.0. Therefore, samples with a GAPDH Cq > 30, indicative of poor quality of the starting RNA sample, were excluded from the analysis.

3.9 Quantification of mitochondrial DNA

Total DNA was purified from cells using a conventional phenol-chloroform extraction method. Relative quantification of mitochondrial DNA (mtDNA) copy number was performed by a real-time PCR method using a CFX96 real-time system (Bio-Rad Laboratories). Quantitative PCR was performed using primers and conditions as previously described (Refinetti et al. 2017).

3.10 Western blot analysis

Western blot analysis was performed on 20 μg of total protein extracts according to the protocol previously described. The following primary antibodies were used:

Primary antibody	**Diluition**
Anti-Flag (Sigma-Aldrich)	1:10.000
Anti-GATA1(D24E4) #4589 (Abcam)	1:1.000
Anti-SDHC (3E2) #H00006391-M01 (Novus Biological)	1:500
Anti-SDHA (2E3GC12FB2AE)2 (Invitrogen\Life-Technologies)	1:10.000
Anti-SDHB (21A11AE7) (Invitrogen\Life-Technologies)	1:10.000
Anti-SDHD #5-34387 (Thermo Fisher)	1:10.000
Anti-SOD2 #1H6 (Thermo Fisher)	1:10.000
Anti-α-actin C-11 (Santa Cruz)	1:1000
Anti -VDAC1 #sc-390996 (Santa Cruz)	1:500
Anti-SOD1 #sc-17767 (Santa Cruz)	1:1000
Anti -DRP1 #D8H5 (Cell Signaling)	1:4.000
Anti-MFN2 #D2D10 (Cell Signaling)	1:5.000
Anti-Glutathione Synthetase # ab124811 (Abcam)	1:20.000
Anti-Glutathione Reductase # ab124995 (Abcam)	1:5.000
Anti-GAPDH #2118 (Abcam)	1:1000

Secondary antibody	Diluition
Anti-mouse (Biorad)	1:10.000
Anti-rabbit (Biorad)	1:10.000
Anti-goat (Santa Cruz)	1:15.000

Table 2. *Antibodies used for Western Blot analysis.*

Filters incubated at 4°C O.N. with primary antibodies were washed thrice with 1X TBS-Tween 20 buffer for 5 min and incubated for 45 min with secondary antibodies conjugated to peroxidase (Bio-Rad Laboratories). Blots were visualized using the ECL Immobilon Western Chemiluminescent HRP-substrate system (Millipore, Darmstadt, Germany) and immunoreactive bands were detected by autoradiography according to the manufacturer's instructions or by ChemiDoc XRS Image System (Bio-Rad Laboratories). Signals were subsequently normalized with antibodies anti-GAPDH (Cell Signaling #2118) or anti α-actin (C-11) (Santa Cruz Biotechnology, Santa Cruz, CA, USA #sc-1615). Quantification of Western blot bands was performed using the ImageJ software.

3.11 Measurement of the enzymatic activity of Complex II Succinate-Ubiquinone Oxidoreductase

Forty-eight hours after transient transfection, K562 cells were harvested and washed three times with 3 mL of cold 1× PBS by centrifugation at 3000× g for 10 min at 4°C. Pellets were resuspended in 200 µL of detergent solution provided by the kit and incubated on ice for 30 min to allow proteins to dissolve. Samples were then centrifuged at 12,000× g for 20 min at 4°C and the supernatant was collected and transferred into a clean Eppendorf tube. Evaluation of protein concentration was performed using the BCA Protein Quantification Kit (Abcam, #ab102536) according to the manufacturer's instructions.
Complex II activity was measured using a Complex II Enzyme Activity Microplate Assay Kit (Abcam). Briefly, lysates (60 µg) were added to a mix (final volume 50 µL) containing incubation buffer to reach the concentration suggested by the manufacturer and hybridized on a 96-well microplate coated with an anti-Complex II monoclonal antibody to recognize and selectively capture the succinate dehydrogenase (SDH) complex; a positive control provided by the manufacturer was used to check hybridization efficiency.
Plates were incubated for 2 h at room temperature according to the manufacturer's instructions. After removing the incubation solution, wells were washed twice with 300 µL of Buffer 1×. Forty microliters of Lipid Mix were added to the wells and the mixtures were incubated for 30 min at room temperature. Finally, 200 µL of substrate (activity solution) was added to each well and the optical density (λ = 600 nm) was measured at room temperature in

a kinetic mode for 60 min on a Synergy H1 Hybrid Multi-Mode Microplate Reader (BioTek, Winooski, VT, USA). Data analysis was carried out according to the manufacturer's instructions.

3.12 Seahorse Assay for Measurement of Cellular Respiration

Extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) were measured in K562 cells transfected either with the empty vector (mock control), GATA- 1_{FL} or GATA-1_S expression vectors using the Seahorse XF Cell Mito Stress test kit on a Seahorse XF24e flux analyzer (Agilent Technologies, Santa Clara, CA, USA) as previously described (Rinaldi et al. 2017). Forty-eight hours after transfection, 6×10^4 cells were seeded in triplicate into poly-lysine-coated cell culture microplates (Agilent Technologies). The flux analysis protocol was as follows: ECAR and OCR were initially measured under basal conditions in XF media (non-buffered DMEM medium supplemented with 10 mM glucose, 2 mM L-glutamine, and 1 mM sodium pyruvate) and after the sequential addition of oligomycin, a complex V inhibitor (1 μM), the mitochondrial uncoupler carbonyl-cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (1 μM) and lastly a combination of the complex I inhibitor rotenone (1 μM) and the complex III inhibitor antimycin A (1 μM). All measurements were normalized to the number of plated viable cells ($6 \times 10^{4)}$. Indices of mitochondrial respiratory function were calculated from the OCR profile: basal OCR (before addition of oligomycin), ATP-linked OCR (calculated as the difference between basal OCR rate and oligomycin- induced OCR rate), and maximal OCR (calculated as the difference of FCCP and rotenone + antimycin A rates). Spare respiratory capacity (SRC) was calculated as the difference between basal and maximal OCR. The results were analyzed in a Seahorse Report Generator (Agilent Technologies) (Panina et al. 2019; Sakamuri et al. 2018).

3.13 Mitochondrial Mass Measurement

Mitochondrial mass was evaluated by cytofluorometry using Intracellular Fixation & Permeabilization Buffer Set (Thermo Fisher Scientific, Inc, # 88-8824-00) according to a two-step protocol for intracellular proteins provided by the manufacturer. Forty-eight hours after transient transfection, K562 cells were harvested and washed twice with cold PBS by centrifugation at 3000 rpm for 10 min at room temperature. Cells were incubated for 30 min with Tom 20 antibody (1:200 dilution Cell Signaling, #42406) at room temperature and protected from light. Cells were then washed and incubated in PBS with a goat anti- rabbit IgG-FITC antibody (1:400 dilution, Alexa Fluor 488 #A11034) for 30 min at room temperature and protected from light. Stained cells were resuspended in an appropriate volume of Flow Cytometry Staining Buffer and the mean fluorescence intensity (MFI) was determined by flow cytometry using an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) and BD Accuri C-

flow software. Net fluorescence signals were evaluated after IgG (Sigma-Aldrich) background subtraction.

3.14 AML Patient Samples

Bone marrow aspiration specimens collected during routine diagnostic tests were obtained from a patient with AML. Informed consent for genetic studies was obtained in agreement with the Declaration of Helsinki. RNA and proteins extraction from bone marrow specimens was performed using the QIAzol (Qiagen) procedure (Riccio et al. 2019).

3.15 Statistical analysis

All data are reported as the mean ± standard deviation of three separate experiments. Statistical differences between mock control and treated cells were calculated using the one-way analysis of variance procedure followed by Dunnett's multiple comparison test, where appropriate. Differences were considered significant when $p < 0.05$ (*) (#) and highly significant when $p < 0.0001$ (**) (##) versus each respective mock control or untreated control group.

4. RESULTS

4.1 Cell viability and apoptosis in cells expressing GATA-1 isoforms

This study was initially aimed to dissect the role of GATA-1_{FL} and GATA-1_S isoforms on cell viability and apoptosis rate, due to the different role played by these isoforms in hematopoietic cell differentiation and proliferation programs. For these purposes, human K562 cells transiently transfected with P3XFlag expression vectors for either GATA-1_{FL} (GATA-1_{FL} cells) or GATA-1_S (GATA-1_S cells) were used as experimental model (Fig. 13).

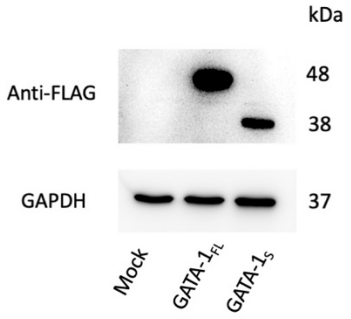


Figure 13: *Western blot analysis (10% SDS-page gels) of the expression levels of GATA-1 isoforms in total protein lysates from K562 cells after transient transfection with either FLAG-tagged GATA-1_{FL} (48 kD), GATA-1_S (38 kD) isoforms or empty vector (mock control).*

As a starting point, cell viability was determined 48 hr after transfection with an MTT assay whereas early and late apoptosis were measured by flow cytometry with the Annexin V/PI detection kit in cells transfected for 48 hr and then treated with the pro-apoptotic drug cisplatin at 10 μM and 20 μM for 16 hr.
MTT results indicated increased cell viability in GATA-1_S cells with respect to GATA-1_{FL} and mock cells, with a higher significant increase 72 hr after transfection whereas GATA-1_{FL} expression was found associated with progressive reduced cell viability even with respect to the mock counterpart (Fig. 14).

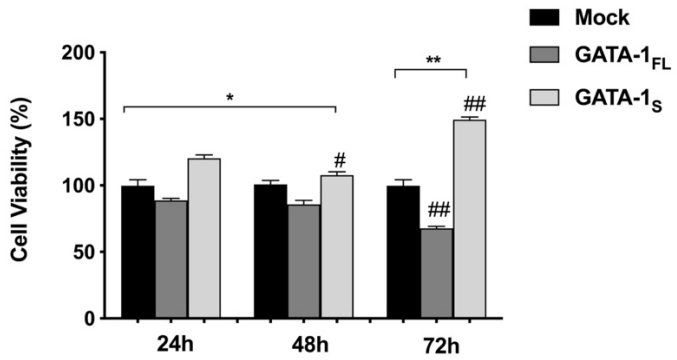


Figure 14: *Cell viability assessed by MTT assay at 24, 48 and 72 hr after transfection in K562 cells over-expressing GATA-1 isoforms and in mock control with GATA-1S cells showing higher cell viability with respect to cells over-expressing full-length isoform and to the mock control. All data shown represent the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when $p < 0.05$ and highly significant when $p < 0.0001$. #$p < 0.05$, ## $p < 0.0001$ versus untreated control group, *$p < 0.05$, **$p < 0.0001$ versus mock control(from Riccio et al. 2019).*

Conversely, evaluation of apoptosis rate under basal condition revealed only slight variations between mock and transfected cells. Cells were then treated with the pro-apoptotic drug cisplatin for 16 hr and results revealed considerable differences in the apoptosis rate between GATA-1_{FL} and GATA-1_S cells, with a strong reduction of both early and late apoptosis in GATA-1_S cells even at the higher tested dose (20 μM) as compared to both mock and GATA-1_{FL} cells (Fig. 15).

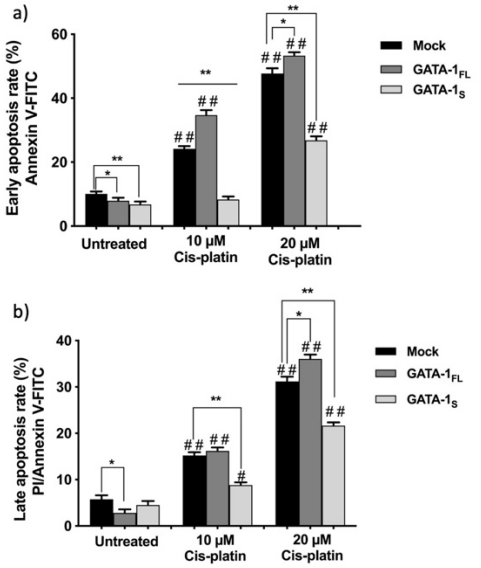


Figure 15: *Flow cytometric analysis of apoptosis rate in K562 cells over-expressing GATA-1 isoforms. a) Early apoptosis rate detected with Annexin V staining in mock control and in K562*

*cells over-expressing GATA-1 isoforms 48 hr after transfection. b) Late apoptosis rate detected with Annexin V/propidium iodide staining in mock control and in K562 cells over-expressing GATA-1 isoforms 48 hr after transfection. Apoptosis was evaluated in untreated cells and in cells treated for 16 hr with 10 and 20 μM cisplatin, respectively. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. #p<0.05, ## p<0.0001 versus untreated control. *p<0.05; **p<0.0001 versus mock control (from Riccio et al. 2019).*

To verify that the pro-apoptotic effect exerted by cisplatin did not involve enhanced ROS production, cytoplasmic ROS levels were evaluated in untrasfected K562 cells after exposure to 10 μM and 20 μM cisplatin, with mendione-treated as positive control of oxidative stress. No variations were found in ROS levels after cisplatin treatment, thus allowing to exclude that the apoptotic mechanism triggered by cisplatin involves changes in oxidative stress conditions (Fig. 16).

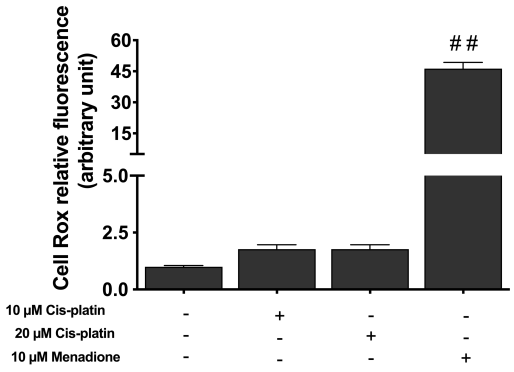


Figure 16: *Cytoplasmatic ROS levels detected by flow cytometry analysis in K562 cells stained with CellRox dye after 10 and 20 μM cisplatin exposure. Menadione treatment (10 μM) was used as positive control for cytoplasmatic ROS production. All data shown represent the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. #p<0.05, ## p<0.0001 versus untreated control (from Riccio et al. 2019).*

4.2 ROS levels and oxidative stress related to different overexpression of GATA-1 specific isoforms

According to the evidence that cellular redox state affects apoptosis susceptibility, experiments were performed to verify if GATA-1_{FL} and GATA-1_S expression could stimulate different oxidative stress and ROS levels.
Total cytoplasmic ROS levels were evaluated using the CellRox probe in transiently transfected K562 cells. Results showed increased levels of cytoplasmatic ROS levels in cells transfected with the two isoforms of GATA-1 with a more marked increase in GATA-1_{FL}. Notably, the enhanced oxidizing

conditions in GATA-1_{FL} cells are consistent with the differentiation program elicited *in vivo* by this GATA-1 isoform in hematopoietic progenitors (Fig. 17).

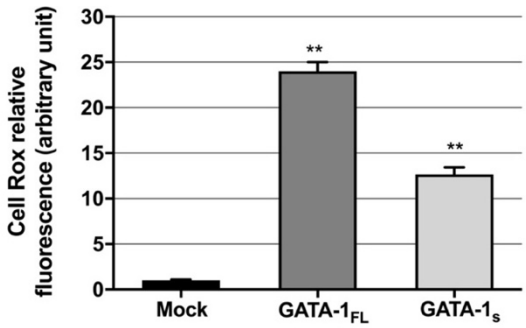


Figure 17: *Cytoplasmatic ROS levels detected by flow cytometry analysis in cells overexpressing GATA-1 isoforms and in mock control after CellRox staining. A dramatic increase of cytoplasmatic ROS in cells over-expressing the full-length isoform of GATA-1. All data are presented as the means ± SD (n = 3 in each group). Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. *p<0.05; **p<0.0001, versus mock control (from Riccio et al. 2019).*

Putative variations in the redox state associated with over-expression of GATA-1 isoforms were also evaluated in the mitochondrial compartment, the major site of ROS production, mainly represented by superoxide anion. Detection of mitochondrial superoxide anion levels with MitoSox staining revealed a marked increase of this compound in GATA-1_S cells with respect to GATA-1_{FL} cells and mock control (Fig. 18).

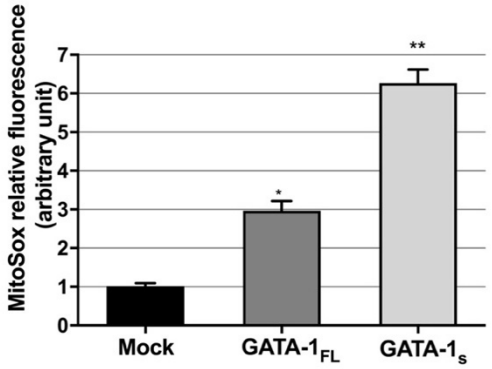


Figure 18: *Mitochondrial superoxide levels detected by flow cytometry analysis in cells overexpressing GATA-1 isoforms and in mock control stained with MitoSoX red mitochondrial superoxide reagent. All data are presented as means ± SD (n = 3 in each group). Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p < 0.05 and highly significant when p<0.0001. *p<0.05; **p<0.0001, versus mock control(from Riccio et al. 2019).*

This finding was apparently incongruent with data indicating elevated cytosolic redox state in these cells since it is expected that a higher ROS content in the cytosol fraction generally reflects elevated ROS production in the mitochondrial compartment (Shadel and Horvath 2015; Sena and Chandel 2012). Therefore, we next asked whether the different superoxide content in these cells could be related to variations in their mitochondrial mass. Cells were thus stained with the Mitotracker green dye that labels only mitochondria that are metabolically active. A dramatic increase of the mitochondrial mass was found in GATA-1_S cells that is in agreement with higher mitochondrial superoxide levels and with the pro-leukemic role hypothesized for GATA-1_S variant as well as recent studies reporting increased mitochondrial mass in myeloid leukemia cells with respect to their normal counterpart (Di Marcantonio et al. 2018; Ye et al. 2015; Farge et al. 2017). On the contrary, GATA- 1_{FL} cells showed reduced mitochondrial mass even with respect to mock cells (Fig. 19a). Consequently, when superoxide levels were normalized to the mitochondrial mass, a higher ratio was found only in GATA-1_{FL} cells that well correlates with cytoplasmatic ROS and mitochondrial superoxide levels in these cells (Fig. 19b).

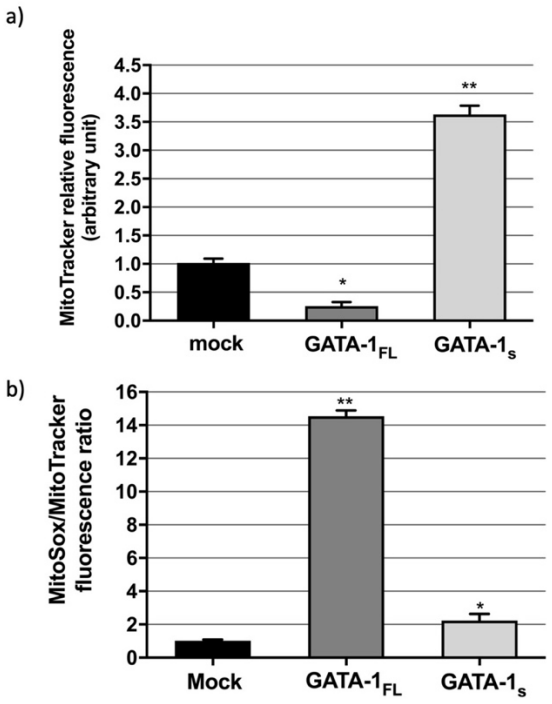


Figure 19: *Mitochondrial mass detected by flow cytometry analysis in cells over-expressing GATA-1 isoforms and in mock control stained with MitoTracker green. a) Total mitochondrial mass detected by flow cytometry analysis in cells overexpressing GATA-1 isoforms and in mock cells stained with MitoTracker green FM reagent. b) Mitochondrial superoxide/mitochondrial mass ratio in cells overexpressing GATA-1 isoforms and in mock control. All data are presented as the means ± SD (n = 3 in each group). Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered*

*significant when p<0.05 and highly significant when p<0.0001. *p<0.05; **p<0.0001, versus mock control (from Riccio et al. 2019).*

To further provide that the superoxide signals revealed by flow cytometric analysis originated from mitochondria, cells incubated with MitoSox, Mitotracker or DAPI were visualized by fluorescence microscopy. As shown in Fig. 20, microscopy images showed the colocalization of the red MitoSox and green Mitotracker fluorescence signals, thus confirming the flow cytometric data. As a whole, these data raise the hypothesis that GATA-1 isoforms differently contribute to mitochondrial remodeling and ROS compartmentalization which, in turn, could be involved in the modulation of the cellular redox environment to support differentiation or proliferation programs in hematopoietic cells as elicited by GATA-1_{FL} and GATA-1_S, respectively.

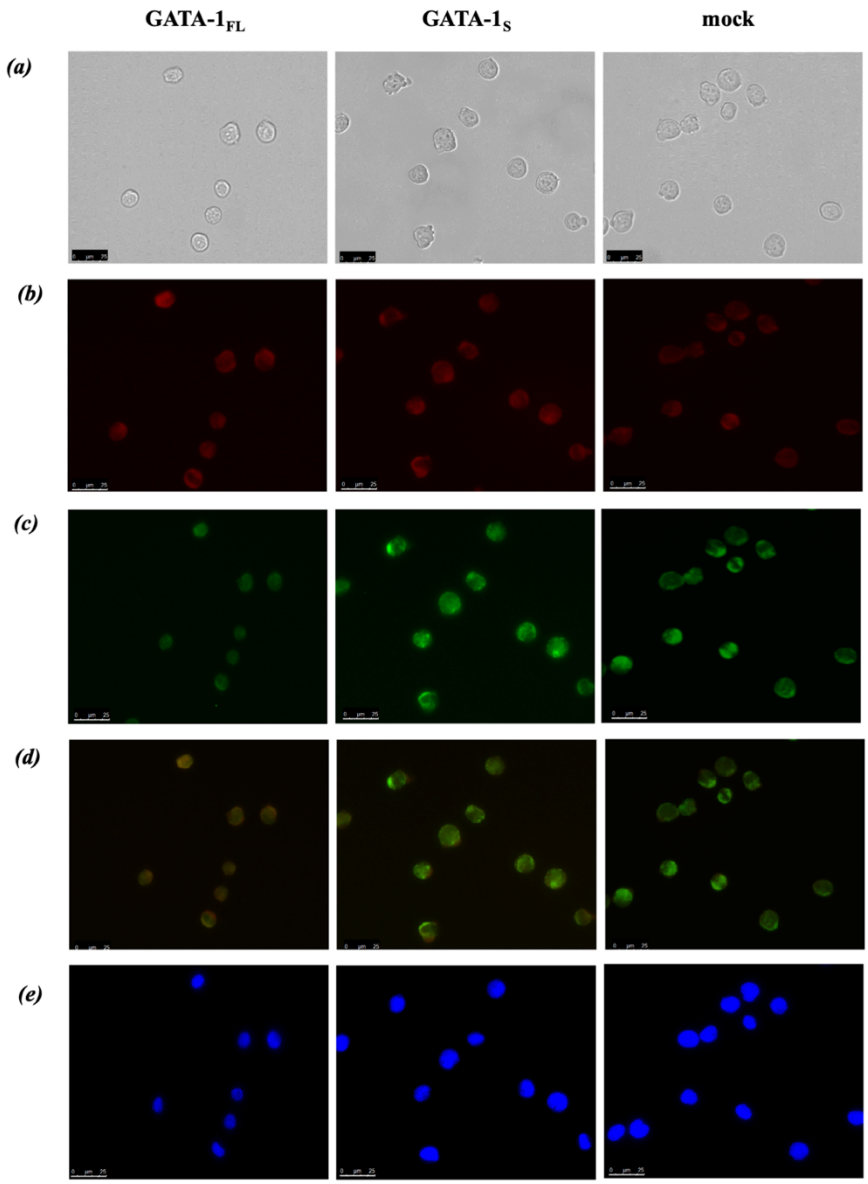


Figure 20: *Fluorescent microscopy images of cells over-expressing GATA-1 isoforms and mock cells. (a) Bright field images; (b) Fluorescence images of K562 stained with MitoSox red mitochondrial dye;(c) Fluorescence images of K562 stained with MitoTracker green FM dye; (d) Merged images; (e) Nuclei stained with 4',6'-diamidino- 2-phenylindole (DAPI). The obtained signals from the merged images (d) show the co-localization of the MitoSox red fluorescence with the Mitotracker green fluorescence, thus confirming the flow cytometric data. This result also clearly showed the prevalence of the MitoSox red fluorescence signal (O_2^- content) in GATA-1_{FL} cells and conversely of the Mitotracker green signal (mitochondrial mass) in GATA-1_S cell (from Riccio et al. 2019).*

4.3 Changes in DNA contents and in mitochondrial dynamics-related proteins

To verify whether the differences in mitochondrial mass found in cells over-expressing GATA-1 isoforms reflected changes in mitochondria size or in their number, quantitation of mtDNA/nuclearDNA ratio was assessed by real-time PCR analysis. Results found that no variations in mitochondrial DNA content were detected in GATA-1_S with respect to the mock control (Fig. 21). This result confirmed that the increased mitochondrial mass in GATA-1_S cells was associated with increased mitochondrial size and excluded the presence of a higher number of mitochondria.

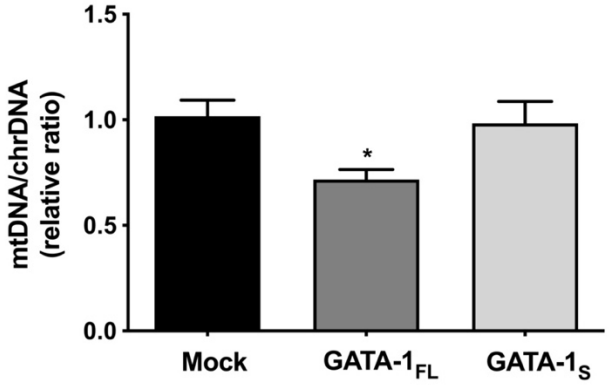


Figure 21: *Changes in mitochondrial DNA contents and in the levels of mitochondrial dynamics-related proteins. This result showed that GATA-1_{FL} cells contain reduced mtDNA relative copy number but conversely, this ratio was almost unaltered in GATA-1_S cells with respect to the mock control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p<0.0001$. *$p<0.05$; **$p<0.0001$, versus mock control(from Riccio et al. 2019).*

Collectively, these data suggest that GATA-1_S expression induces the increase of mitochondrial superoxide anion and triggers mitochondrial remodeling by promoting fusion processes resulting in increased mitochondrial mass. To provide further support to these findings, mitochondrial dynamics-related proteins were also examined. As expected according to the different mitochondrial mass detected in these cells, the expression levels of VDAC1, the most abundant protein in the mitochondrial outer membrane (Gonçalves et al. 2007) were found increased in GATA-1_S cells (Fig. 22).

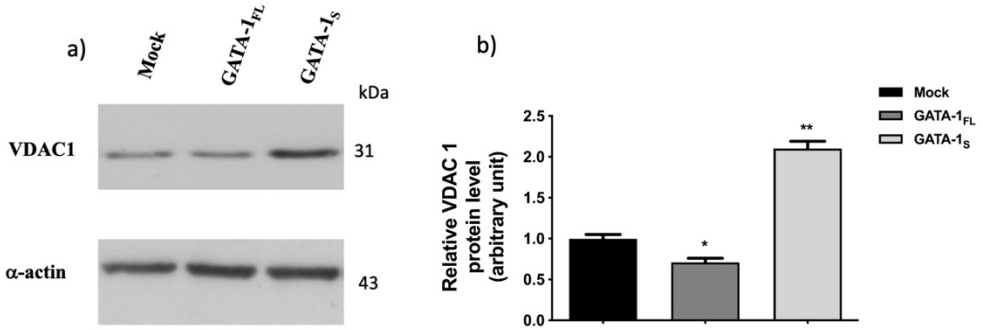


Figure 22: *Changes in mitochondrial DNA contents and in the levels of mitochondrial dynamics-related proteins. a) Western blot analysis (10% SDS-page gels) of VDAC1 expression levels in total protein lysates from mock control and from cells overexpressing GATA-1$_{FL}$ and GATA-1$_S$, respectively. The figure shows representative results of three independent experiment. b) densitometric analysis of Western blot results. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when $p < 0.05$ and highly significant when $p < 0.0001$. *$p < 0.05$; **$p < 0.0001$, versus mock control (from Riccio et al. 2019).*

As regards the mitochondrial fission proteins dynamin-related protein 1 (Drp1) and fusion protein mitofusin 2 (Mfn2) (Ni, Williams, and Ding 2015) Western blot analysis revealed that GATA-1$_S$ expression was accompanied by the over-expression of Mfn2 thus indicating that the increased mitochondrial mass detected in these cells is related to enhanced fusion processes. (Fig. 23). Based on the well-established evidence that mitochondrial fusion help mitigate stress conditions by mixing partially damaged mitochondria (Youle and van der Bliek 2012), these results also raise the hypothesis that GATA-1$_S$ over-expression induces mitochondrial remodeling to reduce the oxidative stress caused by impaired O_2^- production and promote apoptosis resistance. These results thus shed light on a still unexplored role played by GATA-1$_S$ in mitochondrial dynamic remodeling.

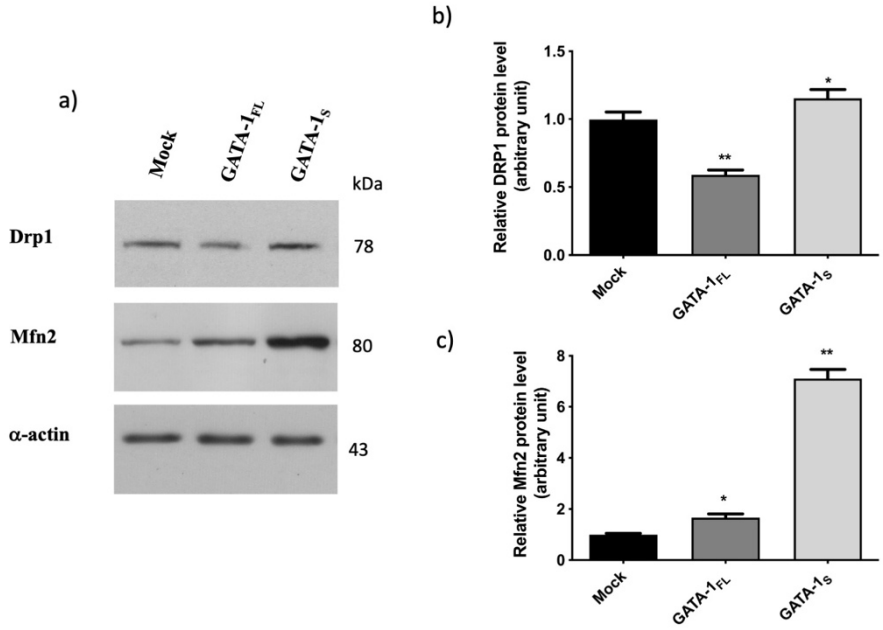


Figure 23: *a) Western blot analysis (10% SDS-page gels) of dynamin-related protein 1 (Drp1) and mitofusin 2 (Mfn2) expression levels in total protein lysates from mock control and from cells over-expressing GATA-1 isoforms. Representative results of three independent experiments are shown; (b,c) Densitometric analysis of Western blot results. For all Western blotting data, band intensities from three independent experiments were quantified and normalized to α-actin used as a loading control. All data were analyzed by one-way ANOVA, followed by Dunnett's multiple comparison test, where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p<0.0001$. *$p<0.05$; **$p<0.0001$, versus mock control (from Riccio et al. 2019).*

4.4 Differential antioxidant capacity in cells expressing GATA-1 isoforms

The close relationship between ROS levels and GATA-1 isoforms prompted us to investigate in these cells possible correlations between different redox states and changes in antioxidant defense capacities. Firstly, the protein levels of SOD1 and SOD2, the cytoplasmatic and mitochondrial superoxide dismutases respectively, were measured. Western blot analysis showed no significant variation in both SOD isoforms in GATA-1_{FL} cells while cells over-expressing GATA-1_S reveled a marked reduction of SOD2 along with a slight increase in SOD1 levels. Notably, in recent years SOD2 has received growing attention as a modulator of cellular apoptosis and as a negative survival factor for cancer cells (Pani et al. 2004). Collectively, these conditions contribute to explain the reduced cytoplasmatic ROS levels and the higher mitochondrial superoxide content detected in these cells (Fig. 24).

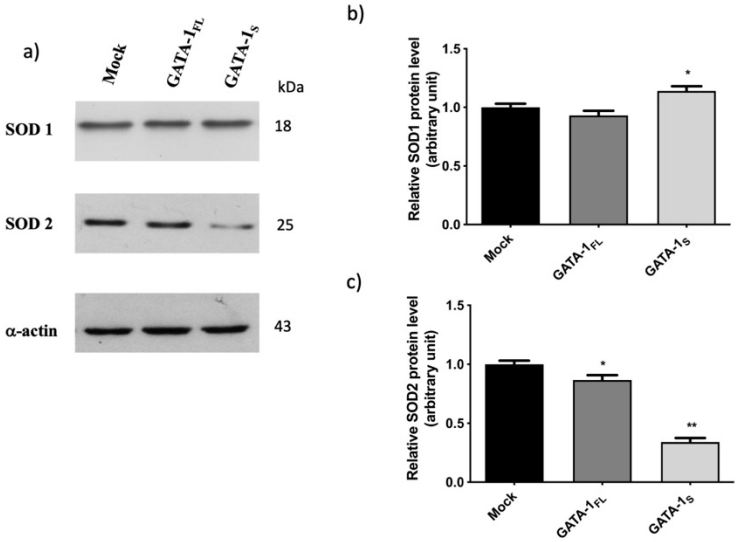


Figure 24: *Evaluation of antioxidant defenses in cells expressing GATA-1 isoforms. a) Western blot analysis (10% SDS-page gels) of the expression levels of Cu/ZnSOD (SOD1) and MnSOD (SOD2) in total protein lysates from mock control and cells over-expressing GATA-1$_{FL}$ and GATA-1$_S$. Each blotting is representative of three independent experiments; b), c) Densitometric analysis of Western blot results. Band intensities were quantified and normalized to α-actin used as loading control. Data are presented as fold-changes relative to the mock control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test, where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p < 0.0001$. *$p<0.05$; **$p<0.0001$, versus mock control (from Riccio et al. 2019).*

Glutathione is another important antioxidant system and, accordingly glutathione redox status is commonly used as indicator of oxidative stress. Evaluation of the glutathione status (total glutathione (GSH+GSSG) and GSH/GSSG ratio) was thus performed in these cells (Fig. 25). GATA-1$_{FL}$ cells showed reduced total GSH levels that were conversely found increased in GATA-1$_S$ cells, thus indicating an enhanced antioxidant capacity in this latter cell type. Furthermore, in GATA-1$_{FL}$ cells a dramatic reduced GSH/GSSG ratio was also found. These findings further reinforce the evidence of a higher oxidative stress status in GATA-1$_{FL}$ cells and of a stronger antioxidant capacity in GATA-1$_S$ cells according to what had been previously hypothesized based on the cytoplasmatic ROS levels detected in these cells.

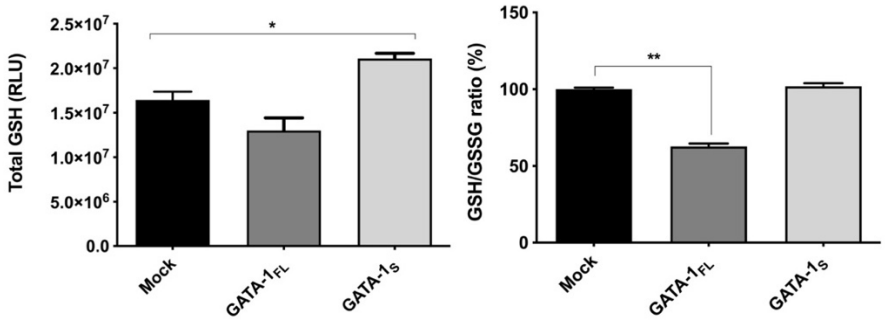


Figure 25: *Total glutathione levels (GSH +GSSG) determined in K562 cells 48 hr after transfection with expression vectors for GATA-1 isoforms or with empty vector (mock). Results represent the net luminescence (as relative luminescence units, RLU) after background subtraction. The mean ± SD of three independent experiments were plotted on the graph; relative GSH/GSSG ratio for mock control or GATA-1$_{FL}$ and GATA-1$_S$ cells were obtained using the following formula: (Net transfected cells total glutathione RLU − Net transfected cells GSSG RLU)/(Net transfected cells GSSG RLU/2). Means ± SD of three independent experiments were plotted on the graph as a relative percentage versus mock control cells. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparison test, where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p < 0.0001$. *$p<0.05$, **$p < 0.0001$ versus mock control (from Riccio et al. 2019).*

With the aim to verify whether GATA-1 isoforms differently contribute to the regulation of GSH metabolism, we evaluated the levels of two enzymes involved in GSH biosynthesis. GSH synthetase (GSS) that catalyzes the second step of *de novo* GSH biosynthesis commonly found upregulated in several cancer types (Bansal and Simon 2018), and GSH reductase (GSR), a flavoprotein enzyme that regenerates GSH from GSSG (Sillar et al. 2019) were also evaluated. Increased levels of GSH synthetase were found in cells over-expressing GATA-1$_S$ compared to both GATA-1$_{FL}$ cells and the mock control. Conversely, GSH reductase levels were found at increased levels in both cell types, even though at slightly higher level in GATA-1$_S$ cells (Fig. 26). These findings further reinforce the evidence of a stronger antioxidant capacity in GATA-1$_S$ cells including enhanced expression of enzymes involved in antioxidant mechanisms as an adaptive response to oxidative stress in GATA-1$_S$ cells that contribute to sustain cell survival under pro-oxidant conditions.

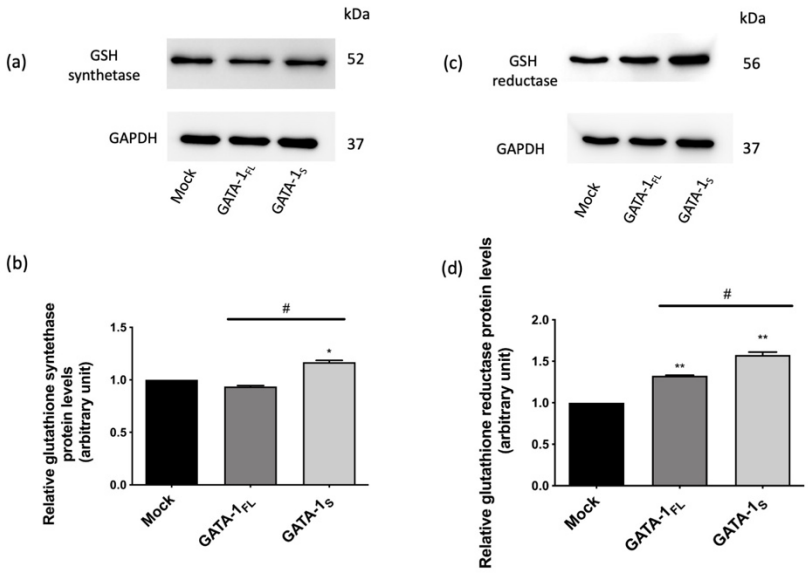


Figure 26: *Expression levels of GSH biosynthetic enzymes. (a) Western blot analysis (10% SDS-page gels) of GSH synthetase expression levels in total protein extracts from mock control and from cells over-expressing GATA-1$_{FL}$ and GATA-1$_{S}$, respectively; (b) Densitometric analysis of Western blot results. The figure shows representative results of three independent experiments; (c) Western blot analysis (10% SDS-page gels) of GSH reductase expression levels in total protein extracts from mock control and from cells over-expressing GATA-1$_{FL}$ and GATA-1$_{S}$, respectively; (d) Densitometric analysis of Western blot results. The figure shows representative results of three independent experiments. Differences were considered significant when $p < 0.05$ and highly significant when $p<0.0001$. # $p<0.05$, GATA-1$_{FL}$ versus GATA-1$_{S}$ * $p<0.05$, ** $p<0.0001$ versus mock control (from Trombetti, Sessa, et al. 2021).*

4.5 Differential response to quercetin treatment in cells expressing GATA-1 isoforms

Different oxidative stress conditions in cells expressing GATA-1 isoforms prompted us to investigate the relationship between variations in viability and apoptosis and the redox state in these cells. To this aim, cells were treated with the flavonoid compound quercetin that may exerts antioxidants effects at low doses or a pro-oxidant activity at higher doses and exposure time. (Akan and Garip 2013; Brisdelli et al. 2007).

Time-course and dose-response experiments were performed in K562 cells treated with 5, 10, 25, 50, 100, and 150 μM quercetin for 3 and 24 hr and cell viability was subsequently assessed with an MTT assay (Fig. 27).

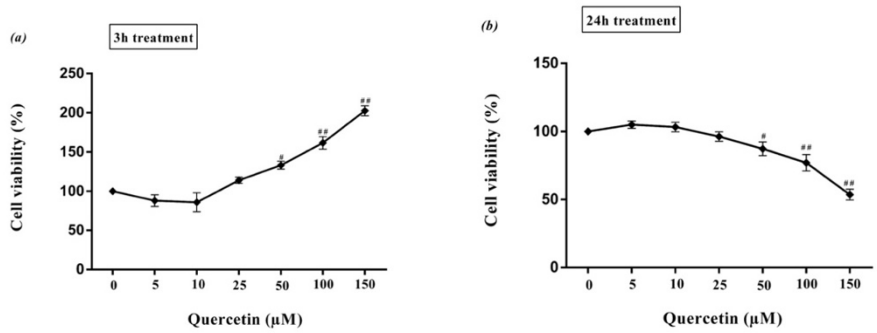


Figure 27: *Evaluation of differential response to quercetin treatment in cells expressing GATA-1 isoforms. (a) Time-course and dose-response to 5, 10, 25, 50, 100, 150 μM quercetin in K562 cells. Cell viability was assessed by the MTT assay after 3 hr (a) and 24 hr exposure (b). Means ± SD of three independent experiments were plotted on the graph as relative percentage versus mock of the untreated control group. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. #p<0.05, ##p<0.0001 versus untreated control group(from Riccio et al. 2019).*

Based on data showing the most effective cell response at 50, 100, and 150 μM treatments, these concentrations were chosen for further experiments. Results showed opposite effects on cell viability in GATA- 1_{FL} and GATA-1_S cells at 3 and 24 hr of treatment. After 3 hr of treatment there was a dose-dependent increased cell viability in both GATA-1_{FL} and GATA-1_S cells. However, whereas at the lowest tested dose (50 μM), GATA-1_S cells displayed enhanced cell viability compared to GATA-1_{FL} cells, at the higher dose (150 μM) there was the opposite effect in favor of GATA-1_{FL} cells (Fig. 28a). On the other side, at longer exposure time (24 hr) all cell types displayed a dose-dependent reduction in cell viability. Interestingly, this effect was significantly greater in GATA-1_S than in GATA-1_{FL} cells (Fig. 28b).

To verify whether variations in cell viability correlate with changes in the apoptotic rate, early and late apoptosis were evaluated by the Annexin V/PI assay in cells exposed to 50 and 150 μM quercetin for 3 and 24 hr. After 3 hr exposure at the higher dose level, a slight increase in the percentage of apoptotic GATA-1_S cells was found. More interestingly, after 24 hr exposure, at both low and high doses of quercetin, GATA-1_S cells showed an enhanced apoptotic rate with respect to the GATA-1_{FL} counterpart (Fig. 28c).

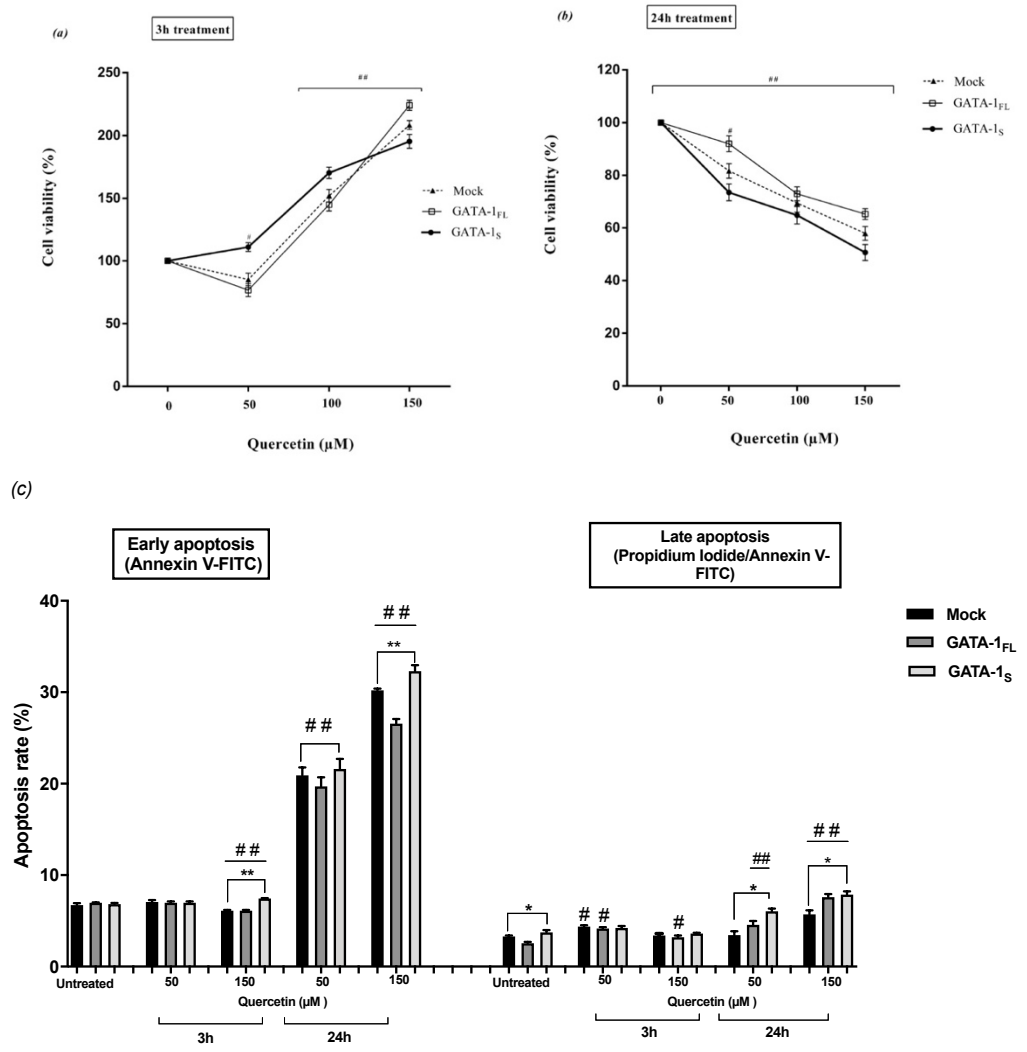


Figure 28: *Cell viability after exposure to 50, 100, and 150 μM quercetin for 3 hr (a) and 24 hr (b) in mock control and in cells over-expressing GATA-1 isoforms 48 hr after transfection. (c) Early and late apoptosis rate in mock control and in cells over-expressing GATA-1 isoforms. Forty-eight hours after transfection, cells were treated for 3 hr and 24 hr with 50 and 150 μM quercetin or with vehicle control (DMSO + PBS). Means ± SD of three independent experiments were plotted on the graph as relative percentage versus mock of the untreated control group. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p<0.0001$. #$p<0.05$, ##$p<0.0001$ versus untreated control group, *$p<0.05$, **$p<0.0001$ versus mock control (from Riccio et al. 2019).*

To further confirm the changes in the apoptotic susceptibility induced by quercetin in GATA-1_{FL} and GATA-1_S cells, apoptosis was also evaluated in cells co-treated with quercetin and 10 μM and 20μM cisplatin for 16 hr. Besides confirming the pro-apoptotic effects of quercetin on both cell types, results

showed enhanced dose-dependent apoptosis rates in GATA-1_S cells with respect to GATA-1_{FL} cells. These results provided experimental evidence that quercetin treatment can revert the resistance to apoptosis in GATA-1_S cells observed when cells are treated with cisplatin alone (Fig. 29).

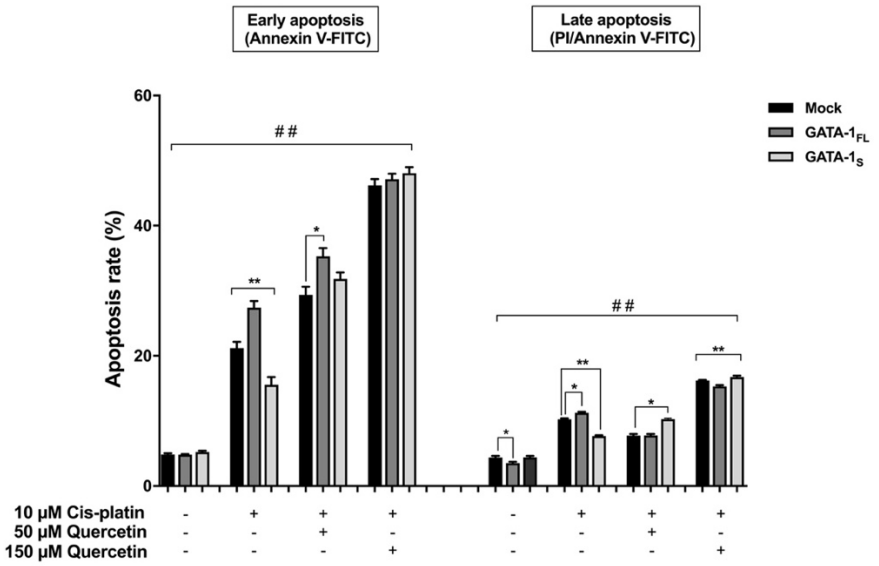


Figure 29: *Early and late apoptosis rate in mock control and in cells over-expressing GATA-1 isoforms after co-treatment with quercetin and cisplatin. The untreated control group was incubated with vehicle control (DMSO+PBS) under the same conditions. All data represent the mean ± SD of three independent experiments. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p < 0.05 and highly significant when p<0.0001. #p<0.05, ##p<0.0001 versus untreated control group, * <0.05, **p<0.0001 versus mock control(from Riccio et al. 2019).*

According to the well-established dual effect of quercetin antioxidant or pro-oxidant activity depending on its different dose and exposure time, these findings were clearly evocative of variations in the cellular redox state induced by high-dose quercetin treatment. To explore this hypothesis, glutathione levels were evaluated in GATA-1_{FL} and GATA-1_S cells treated for 3 and 24 hours with 150 µM quercetin. As shown in Fig. 30, these conditions resulted in a progressive dramatic glutathione depletion in GATA-1_S cells accompanied by a reduced GSH/GSSG ratio. On the contrary, GATA-1_{FL} cells did not show variations in their total glutathione content (Fig. 30a). Furthermore, the short-term quercetin exposure appeared to be consistent with a restored GSH content (Fig. 30b). As a whole, these findings indicate that at short-time and low-dose exposure, quercetin acts a ROS scavenger, thus further reinforcing the antioxidant capacities in GATA-1_S cells, whereas at prolonged time and high-dose exposure it plays a pro-oxidant role, as indicated by the reduced GSH levels. Notably, GATA-1_S cells appear to be more sensible to this treatment and, even more interestingly, the more pronounced depletion in antioxidant defenses elicited by

quercetin under pro-oxidant conditions eventually dramatically reduces their resistance to apoptotic stimuli in these cells.

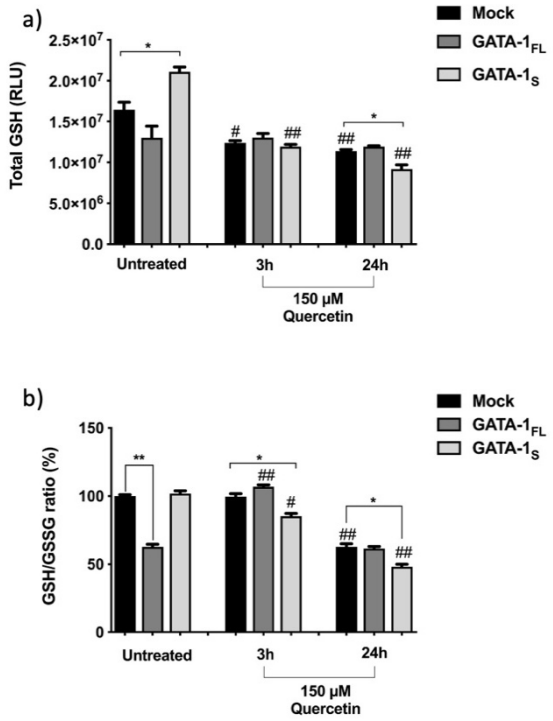


***Figure 30**: a) Total glutathione levels (GSH+GSSG) detected after exposure to 150 µM quercetin or to vehicle control (DMSO+PBS) for 3 hr and 24 hr in K562 cells 48 hr after transfection. Results represent the net luminescence (in RLU) after background subtraction and the mean ± SD of three independent experiments were plotted on the graph; b) Relative GSH/GSSG ratio for mock control or GATA-1$_{FL}$ and GATA-1$_S$ cells treated for 3 hr and 24 hr with 150 µM quercetin or vehicle control (DMSO+PBS). Results were obtained using the following formula: (Net transfected cells total glutathione RLU − Net transfected cells GSSG RLU)/(Net transfected cells GSSG RLU/2). Mean ± SD of three independent experiments were plotted on the graph as relative percentage versus mock of the untreated control group. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p < 0.05 and highly significant when p<0.0001. #p<0.05, ##p<0.0001 versus untreated control group, *p<0.05, **p<0.0001 versus mock control (from Riccio et al. 2019).*

4.6 Correlation between GATA-1 Isoforms and Expression Levels of SDHC Isoforms

The experimental evidence gathered so far, besides highlighting a novel strong correlation between the expression of GATA-1 isoforms, mitochondrial remodeling and oxidative stress, had also raised the hypothesis that metabolic rewiring of mitochondrial oxidative processes might be involved in altered anion superoxide production. To start exploring this issue, respiratory chain complex

II activity (SDH) was investigated in both GATA-1_{FL} and GATA-1_S cells. The rationale behind this choice was based on the recent growing interest on SDH as a potential site of aberrant anion superoxide production (Guzzo et al. 2014). Western blot analysis on protein extracts from GATA-1_{FL} or GATA-1_S cells collected 48 hr after transfection, was used to evaluate the expression levels of the SDH subunits, namely SDHA, SDHB, SDHC, and SDHD. Interestingly, significant variations were found only for the SDHC subunit with higher levels detected only in GATA-1_S cells (Fig. 31). However, when SDHC levels were normalized to the mitochondrial mass, results revealed a marked increased SDHC/mitochondrial mass ratio in GATA-1_{FL} cells and conversely, a dramatic reduced ratio in GATA-1_S cells (Fig. 31c). These data thus were supportive of our starting hypothesis that mechanisms triggered by the two GATA-1 isoforms could differentially involve SDH activity.

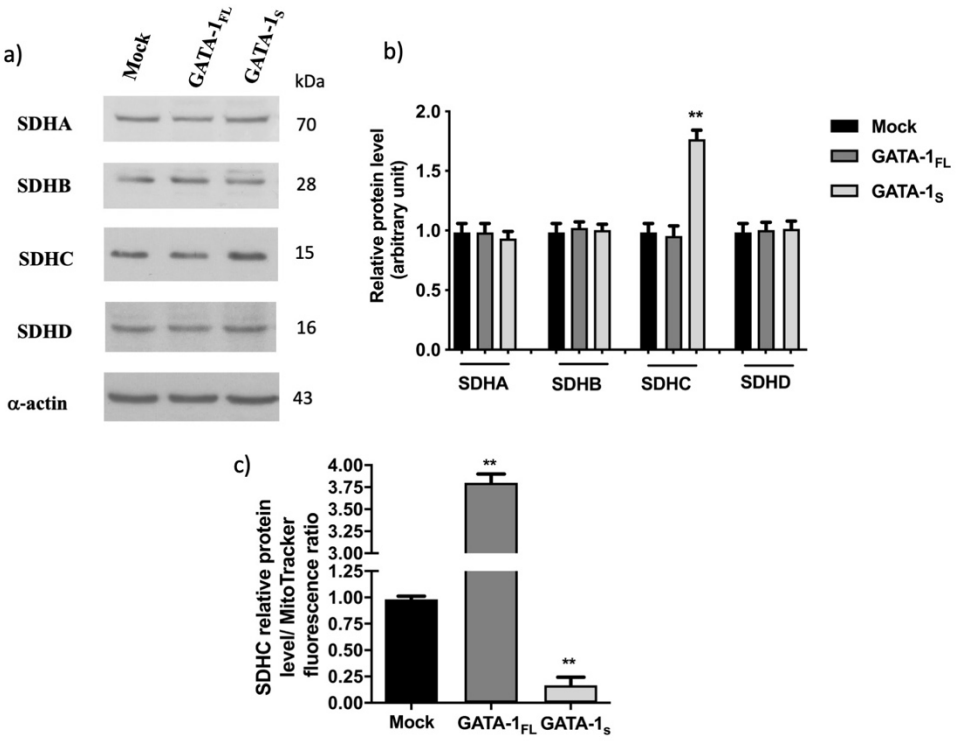


Figure 31: *Evaluation of the expression levels of SDH subunits in K562 cells over-expressing GATA-1 isoforms. (a) Protein levels of SDH complex detected by Western blotting in total protein lysates from mock control and from cells over-expressing GATA-1_{FL} and GATA-1_S, respectively, showing increased levels of SDHC only in GATA-1_S cells. Representative Western blotting (10% SDS-page) of three independent experiments is shown for each SDH subunit and for α-actin used as loading control. b) Densitometric analysis of Western blot results. Band intensities from three independent experiments were quantified and normalized to α-actin. (c) SDHC/mitochondrial mass ratio in mock control and in cells overexpressing GATA-1 isoforms. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when*

*p<0.05 and highly significant when p<0.0001. *p<0.05, **p<0.0001 versus mock control (from Riccio et al. 2019).*

With the aim to further clarify this issue and based on recent data indicating the presence of SDHC alternative splicing isoforms (ASV), namely SDHC Δ3 ASV and SDHC Δ5 ASV, whose over-expression has been recently reported to exert tumorigenic role (Satoh et al. 2015; Moosavi et al. 2020), a high-resolution Western blot analysis was performed in these cells revealing again the increased of SDHC levels and the presence of two bands. (Fig. 32).

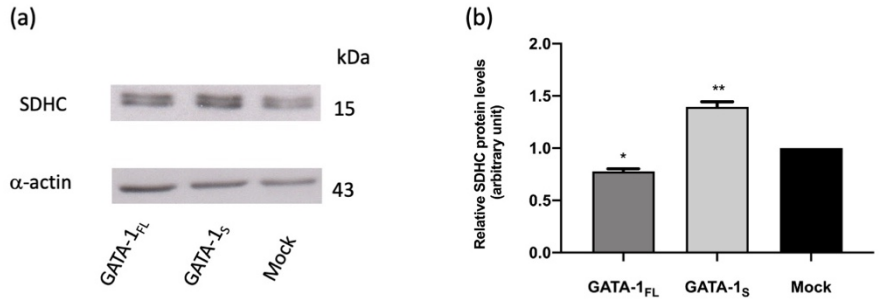


Figure 32: *Western blot analysis (15% SDS-page) on total protein extracts from K562 cells over-expressing GATA-1$_{FL}$ and GATA-1$_S$ isoforms and from a mock control. (a) Representative image of three independent experiments showing the presence of two bands possibly representing different SDHC isoforms. (b) Densitometric analysis of Western blot results showing total SDHC levels markedly increased only in K562 cells over- expressing the GATA-1$_S$ isoform. For each sample, band intensities of the two SDHC signals, taken as a whole, were quantified from three independent experiments, and normalized to α-actin used as a loading control. All data were analyzed for statistical significance by one-way ANOVA, followed by Dunnett's multiple comparison test where appropriate. Differences were considered significant when p < 0.05 and highly significant when p<0.0001. *p<0.05, **p<0.0001 versus mock control (from Trombetti, Sessa, et al. 2021).*

To clarify whether GATA-1 could play a role in the regulation of SDHC expression, an isoform-specific quantitative real-time PCR assay was set-up and used to analyze the full-length SDHC and its two alternative spliced transcripts, Δ3 and Δ5 ASVs.
Primers were designed to selectively amplify four amplicons in separate reactions corresponding to total SDHC transcripts and to the full-length, Δ3 and Δ5 transcripts, respectively. As shown in Fig. 33, RT-PCR analysis, besides confirming at the transcriptional level the Western blot results obtained on total SDHC levels, allowed us to demonstrate that the increase in SDHC associated with GATA-1$_S$ over-expression was mostly due to elevated transcript levels of its Δ5 ASV isoform.

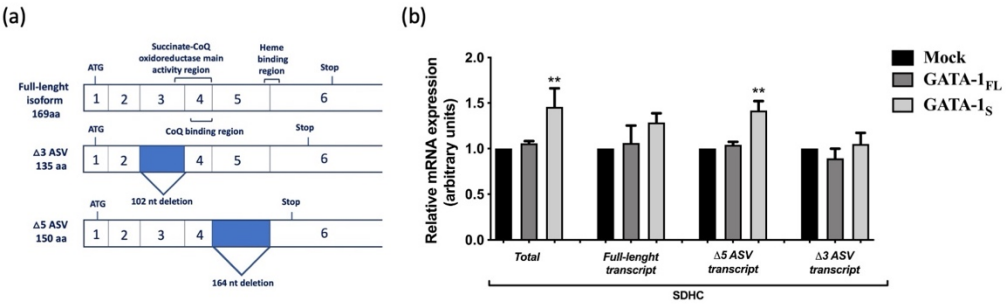


Figure 33: *a) Schematic representation of the alternative splicing mechanism generating SDHC variants (ASVs). Solid boxes and bars indicate the deleted exons and the corresponding protein domains, respectively. b) Quantitative real-time PCR analysis of SDHC mRNA variants in cells over-expressing GATA-1 isoforms and in a mock control. mRNA expression levels were normalized against GAPDH. Results showed increased total SDHC transcript levels in cells over-expressing GATA-1S, thus confirming Western blot analysis. Moreover, transcript-specific amplification revealed that SDHC abnormal expression in these cells was mostly due to the Δ5 ASV transcript. All data represent the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparisons test, where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. * p<0.05, ** p<0.0001 versus mock control (from Trombetti, Sessa, et al. 2021).*

Knock-down of endogenous GATA-1S with a custom-designed siRNA was performed in K562 cells to further confirm results from over-expression studies. Fifteen and thirty percent of GATA-1S silencing was observed after 48 hr exposure to 50 and 100 nM GATA-1S siRNA, respectively (Fig. 34a, b).
No significant reduction was observed in cells over-expressing the GATA-1FL isoform, thus confirming that GATA-1S siRNA specifically targets the shorter GATA-1 transcript. Western blot analysis showed a significant dose-dependent reduction of SDHC in these cells, reaching 50% reduction as compared to a mock control (Fig.34a, c).
The expression levels of the full-length and the two ASV SDHC isoforms were evaluated by quantitative RT-PCR assays after GATA-1S silencing. A more significant dose-dependent reduction was observed for the Δ5 ASV isoform (Fig. 34d). These findings are consistent with the evidence that GATA-1S over-expression is specifically able to drive the abnormal expression of this SDHC Δ5 ASV isoform.

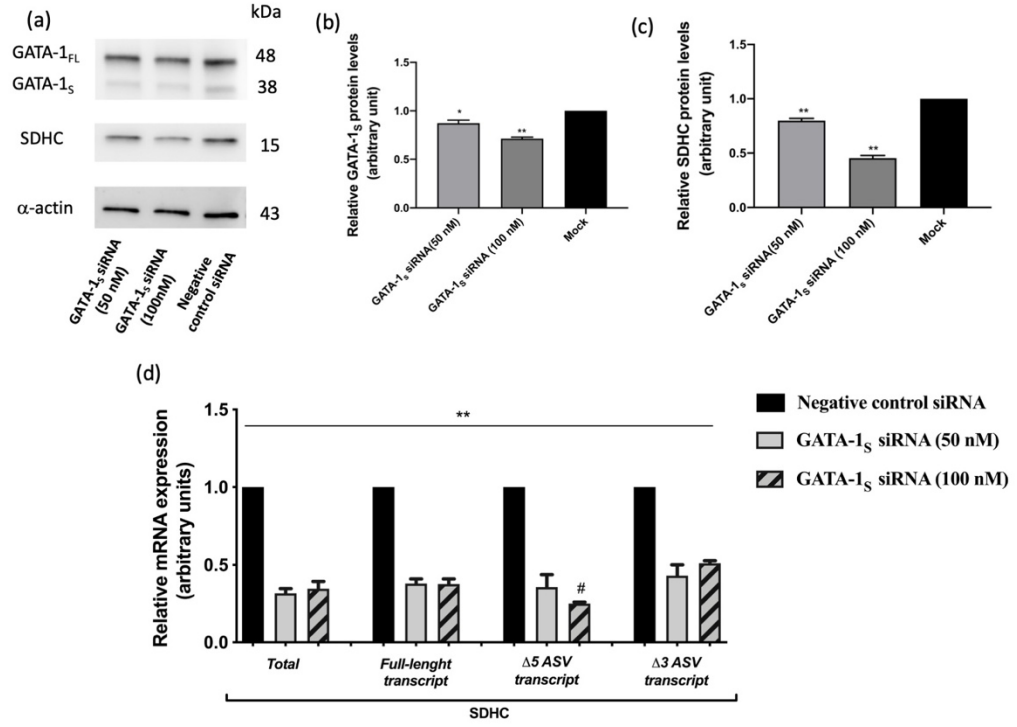


Figure 34: *GATA-1S knockdown experiments: (a) Western blot analysis (10% SDS-page gel) of endogenous levels of GATA-1 isoforms and SDHC after K562 transfection with a custom GATA-1S small interfering RNA (GATA-1S siRNA) at final concentration of 50 and 100 nM. (b) Densitometric analysis of Western blot results of GATA-1S silenced protein. (c) Densitometric analysis of Western blot results for SDHC after specific GATA-1S siRNA transfection. (d) Quantitative real-time PCR analysis of SDHC mRNA variants in K562 cells previously transfected with two doses of specific GATA-1S siRNA. mRNA expression levels were normalized against GAPDH and relative to negative control siRNA transfected cells. Results showed decreased total SDHC transcript levels in cells knocked down for GATA-1S, thus confirming Western blot analysis. In addition, transcript-specific amplification revealed a more significant dose-dependent reduction for the Δ5 ASV isoform of SDHC following GATA-1S silencing. All data represent the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparisons test, where appropriate. Differences were considered significant when $p<0.05$ and highly significant when $p<0.0001$. * $p<0.05$, ** $p<0.0001$ versus negative control; # $p<0.05$ versus lower dose of siRNA transfection (from Trombetti, Sessa, et al. 2021).*

4.7 Effects of GATA-1 Isoforms on Mitochondrial Metabolism

4.7.1 Measurement of SQR Activity

These findings along with literature data indicating that SDHC Δ3 and Δ5 ASVs, act as negative dominant of the SDHC full-length isoform (N. Ishii, Ishii, and Hartman 2006), allowed us to raise the hypothesis that different expression profiles of SDHC isoforms could have an impact on the functional activity of complex II. To assess this hypothesis, complex II succinate-ubiquinone

oxidoreductase activity (SQR) was evaluated in GATA-1_{FL} and GATA-1_S cells. Results showed that SQR activity significantly decreased to about 65% in GATA-1_S cells as compared to the mock control (Fig. 35). Conversely, a slight increase in SQR activity was found associated with GATA-1_{FL} over-expression compared to the mock control (Fig. 35). Notably, these results were in agreement with literature data indicating reduced SQR activity in cells over-expressing the SDHC Δ5 ASV variant (Satoh et al. 2015). Therefore, the reduced SQR activity observed in GATA-1_S cells over-expressing SDHC Δ5 may reflect impaired assembly of the SDH tetramer (Fig. 10) All these data are in agreement with recent compelling evidence indicating that complex II dysfunctions can also generate high levels of superoxide and therefore contribute to tumorigenesis. These alterations may be due either to the overexpression of one or more subunits of this complex or to point mutations or splice variants that cause disassembly of complex II and consequently a decrease in SQR activity. (Panina et al. 2019; Farge et al. 2017; Raimondi, Ciccarese, and Ciminale 2020).

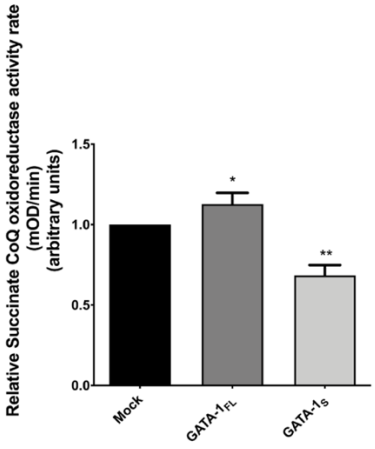
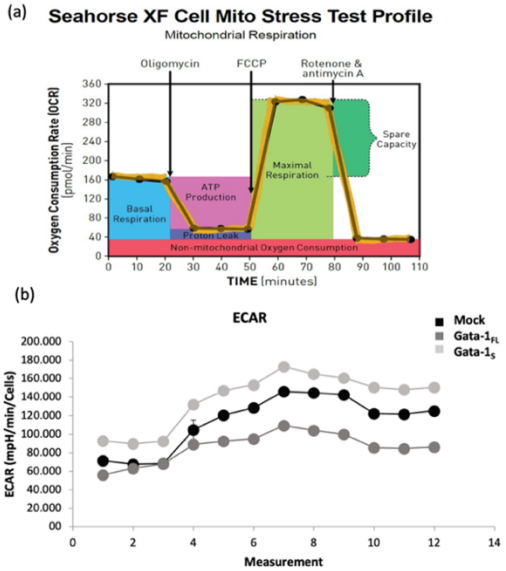


Figure 35: *SQR activity detected on total cell lysates is expressed as OD absorbance/min/mg total protein. Data represent mean ± SD from three independent experiments. Differences were considered significant when *p<0.05 and highly significant when ** p<0.0001 versus mock control (from Trombetti, Sessa, et al. 2021).*

4.7.2 Evaluation of Cellular Energy Metabolism

Given the role played by complex II in oxidative metabolism, we next asked whether the expression of GATA-1 isoforms could be related to differences in mitochondrial oxidative metabolism. To verify this hypothesis, MitoStress tests were performed on a Seahorse flux analyzer in collaboration with Professor Antonio Feliciello's laboratory at our Department. These experiments allows to measure real-time mitochondrial respiration parameters in living cells (Fig. 36a) thus providing information regarding energy metabolism in terms of extracellular acidification (ECAR), basal and maximal respiration, spare

respiratory capacity (SRC), proton leak, and ATP production (Kirschberg et al. 2020; Marchetti et al. 2020).
In GATA-1_S cells, ECAR measurements that are indirectly indicative of glycolytic flux (Nelson et al. 2021), revealed higher ECAR levels and a rightward ECAR shift relative to oxygen consumption rate (OCR) only in GATA-1_S cells (Fig. 36b-d).
This result indicated that GATA-1_S cells utilize glycolysis at higher rate than mitochondrial oxidative phosphorylation to meet energy requirements. As regards mitochondrial respiration, results indicated that cells over-expressing GATA-1_S show an about two-fold increase in their basal respiration compared to GATA-1_{FL} cells and only a slighter increase compared to control; as far as other mitochondrial respiratory parameters are concerned, statistically lower SCR accompanied by a significant reduction in proton leak, maximal respiration and ATP production were observed in GATA-1_{FL} cells compared to both GATA-1_S and mock control (Fig. 36e-h). Conversely, mitochondria in GATA-1_S cells showed enhanced basal and maximal respiration rates, but also higher proton leak, a sign of mitochondrial damage that underlines low coupling efficiency. Additionally, in apparent contrast with these data, enhanced ATP production was also detected in GATA-1_S cells, suggestive of a more efficient OXPHOS process (Fig. 36h).



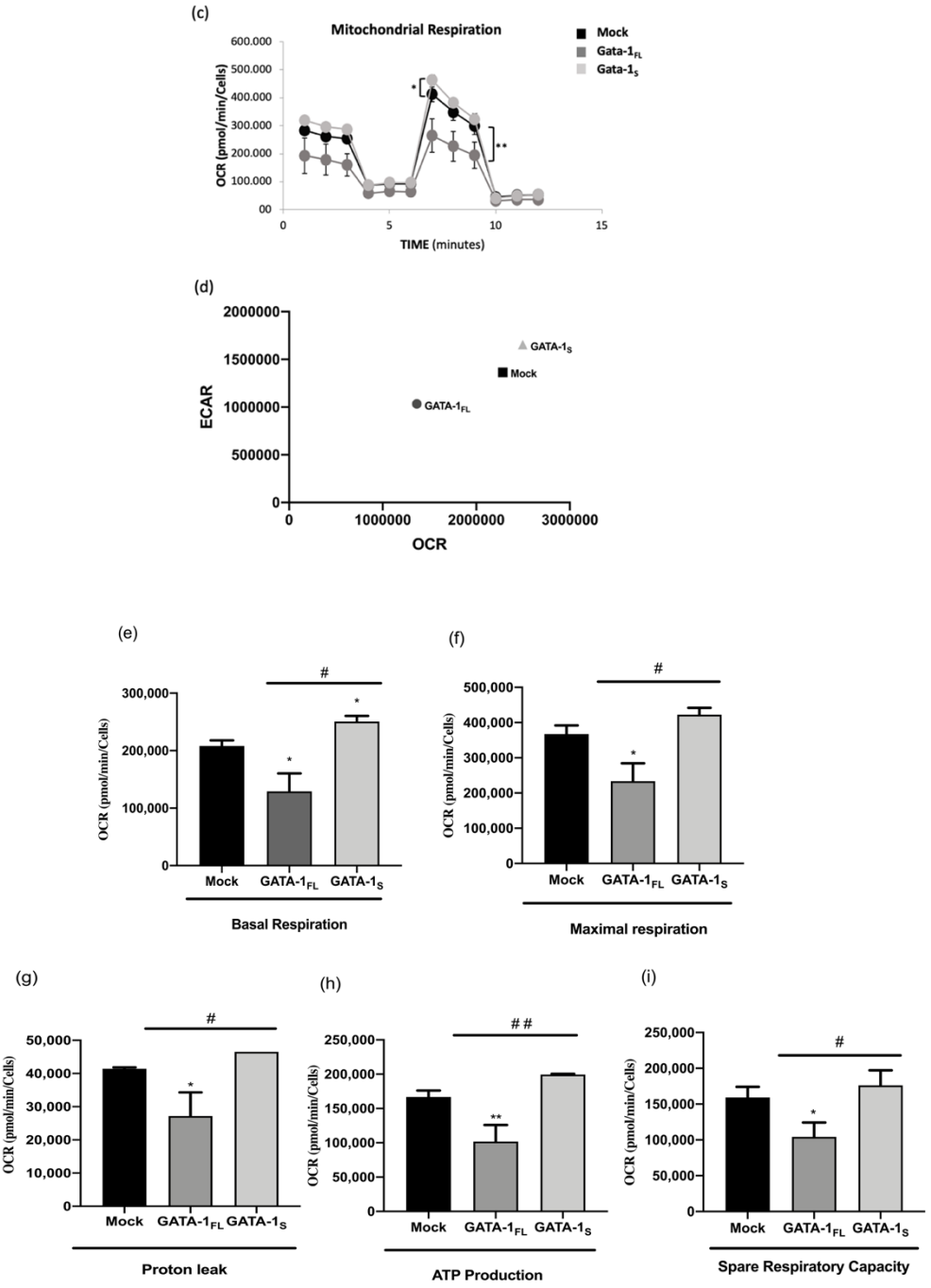


Figure 36: *Mitochondrial respiration rates measured with a Seahorse XFe assay in K562 cells overexpressing GATA-1 isoforms. (a) Schematic description of the experimental procedure; (b,c) variations in extracellular acidification (ECAR) and oxygen consumption rate (OCR, pmol/min/ng/mL) that were found increased in cells over-expressing GATA-1S and decreased in response to GATA-1FL over-expression as compared to the mock control. (d) Relationship between ECAR and OCR in intact cells under basal conditions. Data are presented as mean ± SEM. (e–i). Mitochondrial respiration rates measured by a Seahorse XFe assay in K562 cells overexpressing GATA-1 isoforms. Evaluation of basal (e) and maximal (f) respiration rates,*

*proton leak (g), ATP production (h) and spare respiratory capacity (i) showing that GATA-1$_{FL}$ over-expression is accompanied by reduced proton leak and ATP production. Conversely, over-expression of GATA-1$_S$ is associated with higher respiration rate, enhanced proton leak, ATP production and spare respiratory capacity. These data are suggestive of GATA-1$_S$ cells mainly being dependent on mitochondrial oxidative processes with respect to the full-length isoform. Data represent mean ± SD from three independent experiments. Differences were considered significant when p<0.05 and highly significant when p<0.0001. # p<0.05, ## p<0.0001 GATA-1$_{FL}$ versus GATA-1$_S$, * p<0.05, ** p<0.0001 versus mock control (from Trombetti, Sessa, et al. 2021).*

With the aim to clarify these conflicting findings, respiration parameters were normalized to the size of the mitochondrial network that had been assessed through Tom20 levels, a mitochondrial inner membrane marker protein (Fonseca et al. 2019) that consistently with our previous findings, were indicative of enhanced mitochondrial mass in GATA-1$_S$ cells oppositely to the reduced mass detected in GATA-1$_{FL}$ cells (Fig. 37).

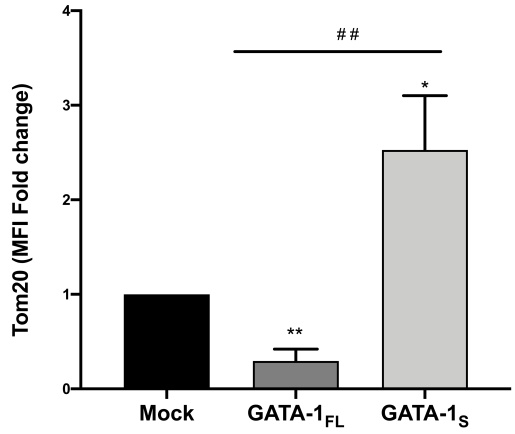


Figure 37: *Total mitochondrial mass in cells over-expressing GATA-1 isoforms. Flow cytometry analysis was performed in fixed and permeabilized cells stained with Tom20 antibody 48 h after transfection. Results are indicative of increased mitochondrial network in cells over-expressing GATA-1$_S$, thus confirming our previous findings. Data represent mean ± SD from three independent experiments. Differences were considered significant when p<0.05 and highly significant when p<0.0001. ## p<0.0001 GATA-1$_{FL}$ versus GATA-1$_S$, * p<0.05, ** p<0.0001 versus mock control(from Trombetti, Sessa, et al. 2021).*

Therefore, when normalized to the size of the mitochondrial network, we found that the indices of mitochondrial respiration in GATA-1$_S$ cells were about 4-fold lower compared to either GATA-1$_{FL}$ or mock control (Fig. 38).

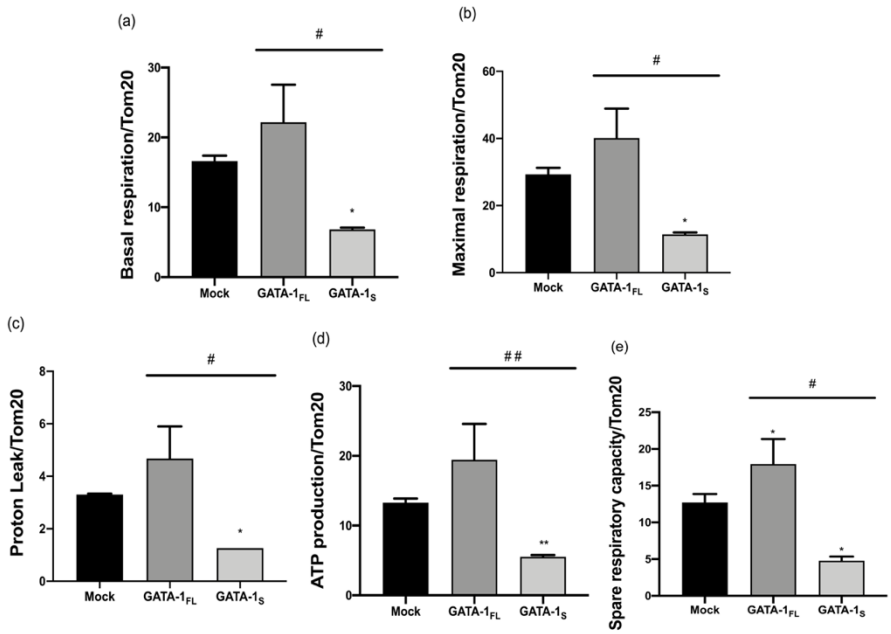


Figure 38: *Cellular respiration rates related to mitochondrial mass in cells over-expressing GATA-1 isoforms. Basal (a) and maximal respiration (b) rates, proton leak (c), ATP production (d), and spare respiratory capacity (e) related to mitochondrial mass. Data represent mean ± SD from three independent experiments. Differences were considered significant when $p < 0.05$ and highly significant when $p<0.0001$. # $p<0.05$, ## $p<0.0001$ GATA-1_{FL} versus GATA-1_S, * $p<0.05$, ** $p<0.0001$ versus mock control (from Trombetti, Sessa, et al. 2021).*

This result suggests that at least a part of the larger mitochondrial network in GATA-1_S cells appears to be damaged and, therefore, is unable to contribute to the oxidative metabolism. These findings thus provided evidence that the energy metabolism in cells over-expressing GATA-1_S is more dependent on glycolysis than on mitochondrial respiration due to reduced OXPHOS efficiency. To further investigate the possible molecular mechanisms correlating GATA-1_S with enhanced glycolytic flux, the expression levels of HIF-1α was examined in cells over-expressing GATA-1 isoforms. As shown in Fig. 39, high levels of the HIF-1α transcript were observed only in cells over-expressing GATA-1_S, thus suggesting that GATA-1 can promote anaerobic metabolism though increased expression of HIF-1α.

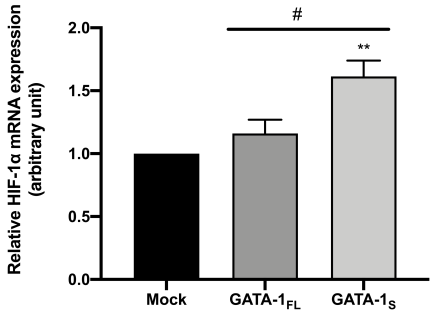


Figure 39: *Quantitative real-time PCR analysis of HIF-1α transcript levels in cells over-expressing GATA-1 isoforms and in a mock control. mRNA expression levels were normalized against GAPDH. Results showed more significant increment of HIF-1α transcript levels in cells over-expressing GATA- 1S, with respect to both GATA-1FL and the mock control. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparisons test, where appropriate. Differences were considered significant when p<0.05 and highly significant when p<0.0001. # p<0.05, GATA-1FL versus GATA-1S, ** p<0.0001 versus mock control (from Trombetti, Sessa, et al. 2021).*

4.8 Expression levels of SDHC and GATA-1 isoforms in an AML patient

Finally, to evaluate possible clinical implications to our findings, bone marrow samples of a patient with AML were collected at different stages of the disease to evaluate GATA-1 and SDHC isoforms levels. Dramatically elevated levels of both GATA-1S and SDHC were detected at diagnosis, during the acute phase of the disease, that were completely normalized at remission (Fig. 40a) in contrast with GATA-1FL that appears to maintain more stable and lower levels in the course of the disease, thus further supporting the specific pro-leukemic role of GATA-1S in myeloid cells as so far hypothesized (Crispino 2005; Halsey et al. 2012; Khan, Malinge, and Crispino 2011). Notably, the increased SDHC levels detected in this patient at diagnosis resulted to be mostly due to both SDHC Δ3 ASV and SDHC Δ5 ASV with respect to the full-length isoform. Even more interesting, the reduced SDHC level during the post therapy phase (at the remission), was mostly related to a dramatic reduction of the two SDHC ASV isoforms (Fig. 40b).

Noteworthy, although it was not possible to evaluate mitochondrial mass and ROS levels in these samples these results are consistent with results gathered in K562 cells since, even in the patient samples, over-expression of GATA-1S (Fig. 40b) correlates with increased expression levels of these two SDHC ASVs.

Furthermore, HIF-1α mRNA levels (Kocabas et al. 2015),were also detected in these AML samples. Results showed significantly increased HIF-1α levels concomitant with GATA-1S over-expression (Fig. 40c) thus further corroborating the relationship between GATA-1S and HIF-1α levels detected in K562 cells. Notably, collectively, these findings could contribute to clarify the

molecular mechanisms underlying the energy metabolic changes observed in cells over-expressing GATA-1S.

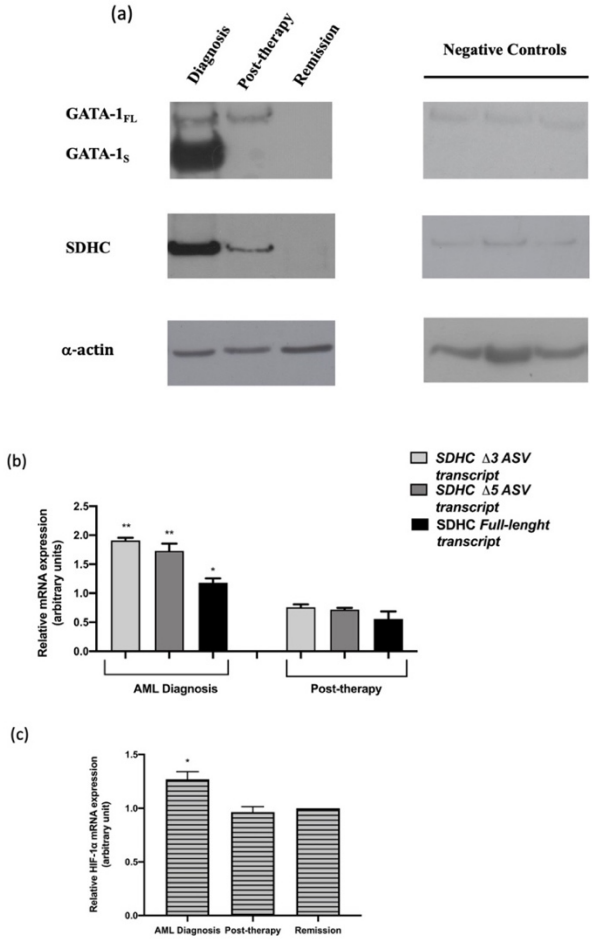


Figure 40: (a) *Evaluation of the expression levels of SDHC and GATA-1 isoforms in a patient with AML. Protein levels of GATA-1 and SDHC detected by Western blotting in total protein lysates from bone marrow biopsies of a patient with AML at different stages of the disease and three healthy negative controls, showing prevalent expression of GATA-1S isoform and elevated SDHC levels during the acute phase of the disease and their normalization at remission. Representative Western blots are shown for GATA-1, SDHC and α-actin used as a loading control; (b) Quantitative analysis of SDHC mRNA variant transcripts from bone marrow specimens of an AML patient at diagnosis and post-therapy stages relative to the remission values. mRNA expression levels were normalized against GAPDH; (c) Quantitative real-time PCR analysis of HIF-1α transcript levels, normalized against GAPDH, in bone marrow samples obtained from the AML patient at diagnosis, post-therapy and remission stages. Results showed significant increment of HIF-1α transcript levels at the diagnosis stage with respect to post-therapy and remission stages. All data represent the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA, followed by Dunnett's multiple comparisons test, where appropriate. Differences were considered significant when $p < 0.05$ and highly significant when $p < 0.0001$. * $p < 0.05$, ** $p < 0.0001$ versus control(from Trombetti, Sessa, et al. 2021).*

5. DISCUSSION

A refined equilibrium between self-renewal, proliferation, differentiation and survival of stem cells is necessary to maintain hematopoietic homeostasis and their progeny. Each process is supported by the balanced expression of several specific genes, regulated by different transcription factors and, in this context, the GATA-1 factor plays a key role in regulating the expression of a large set of hematopoietic-related genes (Hasegawa and Shimizu 2017; Garnett, Cruz Hernandez, and Vyas 2020). Therefore, not surprisingly, its dysregulated expression is emerging as a key factor in malignant hematopoiesis (Crispino 2005; Halsey et al. 2012; Khan, Malinge, and Crispino 2011).

Two isoforms encoded from the same gene through alternative splicing are already known: the full-length GATA-1 isoform (GATA-1_{FL}) and its shorter isoform (GATA-1_S), lacking the N-terminal transactivation domain. These two isoforms play opposite roles in the differentiation and proliferation processes, with the GATA-1_{FL} promoting the terminal differentiation and GATA-1_S mainly involved in the maintenance of the proliferative potency of hematopoietic precursors (Riccio et al. 2019; Garnett, Cruz Hernandez, and Vyas 2020; Kaneko et al. 2012; Tremblay, Sanchez-Ferras, and Bouchard 2018).

These two variants are physiologically expressed at an optimized balanced ratio required for normal hematopoiesis. On the contrary, unbalanced GATA-1_{FL}/GATA-1_S expression with a prevalent expression of GATA-1_S has been found associated with several hematopoietic disorders including different acute and chronic myeloid leukemia subtypes where elevated GATA-1_S levels are recognized as a poor prognostic factor (Halsey et al. 2012; Lentjes et al. 2016; Xu et al. 2014). However, although several reports emphasize the pro-leukemic role of this isoform in hematological malignancies, mechanistic details still need clarification. This study was designed with the aim to contribute to decipher the role of GATA-1_S in malignant hematopoiesis. Research started by investigating the role of GATA-1 isoforms on cell viability and apoptosis rate using K562 cells transiently transfected with expression vectors for GATA-1_{FL} (GATA-1_{FL} cells) or GATA-1_S (GATA-1_S cells) isoforms as experimental model. Results showed enhanced cell viability and resistance to pro-apoptotic stimuli in GATA-1_S cells with respect to the full-length counterpart. Next, experiments were performed to verify if there was a correlation between ROS production and variations in apoptosis sensitivity observed in these cells. Variations in ROS levels and in their intracellular compartmentation were found associated with the expression of specific GATA-1 isoforms: cytosolic ROS resulted to be dramatically increased in both cell types whereas, unexpectedly, increased mitochondrial superoxide levels were found only in GATA-1_S cells. These data were apparently incongruent since mitochondrial superoxide is an important source for cytoplasmic ROS (Brand 2016; Shadel and Horvath 2015) and a higher superoxide content generally corresponds to increased cytoplasmic ROS levels. However, an explanation to these apparently abnormal findings was provided when superoxide levels were normalized over the mitochondrial mass

that was found increased in GATA-1_S cells, revealing an elevated superoxide/mitochondrial mass ratio only in GATA-1_{FL} cells. In this case, the higher mitochondrial superoxide concentration in this latter cell type well correlates with the increased cytoplasmic ROS levels detected in these cells with respect to the GATA-1_S counterpart. To clarify whether the differences in mitochondrial mass found in cells over-expressing GATA-1 isoforms underlie mitochondrial fission and/or fusion processes, mtDNA content was evaluated along with the levels of canonical mitochondrial dynamics-related proteins, including VDAC1, a component of the outer mitochondrial membrane, the fission protein Drp1 and the fusion protein Mfn2. Results were clearly consistent with enhanced rates of mitochondrial fusion processes in GATA-1_S cells, thus discoling novel roles played by GATA-1 isoforms in dynamic remodeling of the mitochondria network.

These findings raised the possibility that the increased mitochondrial network in GATA-1_S cells could contribute to modulate redox signaling to promote cell proliferation and resistance to pro-apoptotic stimuli. To investigate this hypothesis, mechanisms involving mitochondria redox metabolism and antioxidant defenses, including superoxide dismutase levels and glutathione redox state, were examined. In agreement with the mitochondrial superoxide content detected in these cells, GATA-1_S cells showed a slight increase in the cytoplasmatic superoxide dismutase (SOD1) levels and reduced levels of its mitochondrial isoform (SOD2). This result is of relevance in particular in light of recent literature reporting reduced SOD2 expression levels in acute and chronic myeloid leukemia patients (Ciarcia et al. 2010; Girerd et al. 2018; Y.-H. Wang et al. 2014). Also, it is noteworthy that SOD2 plays a crucial role in regulating ROS signaling by controlling the conversion of superoxide anion to other reactive oxygen species that can reach the cytoplasm (Y. Wang et al. 2018). Therefore, these findings are consistent with the increased mitochondrial superoxide levels and the corresponding reduced cytoplasmatic ROS found in GATA-1_S cells.

Variations were also found in GSH content, with increased GSH levels, mainly in its reduced form, in GATA-1_S cells. Enhanced expression of enzymes involved in glutathione metabolism was detected in GATA-1_S cells that reinforce the antioxidant defenses in these cells. Collectively, these findings are consistent with a stronger antioxidant capacity in GATA-1_S cells and provide further insights into the mechanisms triggered by GATA-1_S to escape excessive ROS production.

Next, to investigate whether changes in cell viability and apoptosis rate detected in these cells were directly related to their redox states, cells were treated with the flavonoid quercetin that could exert anti or pro-oxidant role in several cell lines (Akan and Garip 2013; Brisdelli et al. 2007). Cell viability and apoptosis rate were evaluated after short- and long-term treatments (3 and 24 hr) with increasing doses of quercetin. Under antioxidant conditions, with quercetin acting as ROS scavenger, GATA-1_S cells displayed a higher rate of cell viability compared to GATA-1_{FL} cells whereas under pro-oxidant conditions cell viability

was dramatically reduced, particularly in GATA-1_S cells, probably due to a more substantial depletion in their GSH content, a common feature of cell systems with enhanced antioxidant defenses that, like Achilles' heel, make them more sensible to oxidative stress conditions. Pro-oxidant treatment induces further increase in ROS levels beyond that already produced by the malignant cell, either by depleting antioxidant defenses or augmenting ROS production. Therefore, it is conceivable that treatment-induced oxidative stress combined with the intrinsic stress already present in the malignant cell can enhanced lipid peroxidation, oxidation of redox-sensitive residues within proteins, and DNA oxidation resulting in base-transversion and DSBs (Ferraresi et al. 2005; Cockfield and Schafer 2019; He et al. 2017; Hole, Darley, and Tonks 2011). Even more interestingly, in our hands the more pronounced depletion in antioxidant defenses elicited by quercetin under pro-oxidant conditions turned out to dramatically revert their resistance to apoptotic stimuli in GATA-1_S cells. Therefore, this approach provides strong experimental evidence to the relationship between cell viability, and apoptosis rates and changes in the redox states in these cells.

Differences in mitochondrial anion superoxide production also raised the hypothesis that metabolic rewiring of mitochondrial oxidative processes might be involved in these processes. To verify this hypothesis, respiratory chain complex II (SDH) activity was investigated in GATA-1_{FL} or GATA-1_S cells based on preliminary data from our lab suggesting different oxidative redox states of cytochrome b560, the heme moiety of complex II, as well as on a growing interest regarding the role of SDH as a tumor suppressor factor and the relationship between complex II dysregulation and tumorigenesis consequent to chronic ROS elevation and impaired regulation of apoptosis (Rasheed and Tarjan 2018; Dalla Pozza et al. 2020; Rutter, Winge, and Schiffman 2010). Accordingly, germline mutations in SDH genes resulting in impaired SDH activity have been found in several tumor types including pheochromocytoma, paraganglioma, gastrointestinal carcinoma, renal cell carcinoma, thyroid carcinoma, neuroblastoma, and breast cancer along with altered SDH epigenetic and post-translational mechanisms of regulation (Amar et al. 2021; Andrews et al. 2018; Slane et al. 2006; N. Ishii, Ishii, and Hartman 2006; De Sousa et al. 2020). Furthermore, the oncogenic activity of these mutations has been associated with a high production of O_2^- that may be responsible for the genomic instability. Analysis of the expression levels of the SDH subunits, revealed significant variations only for the SDHC subunit showing higher levels in GATA-1_S cells. This finding is of great interest according to recent reports indicating that SDHC overexpression can inhibit succinate-coenzyme Q reductase, without compromising the oxidation activity of succinate to fumarate (Grimm 2013). Inhibition of electron transfer to ubiquinone would hinder oxygen and, in turn, lead to the production and accumulation of superoxide anion (Quinlan et al. 2012). However, normalization of SDHC protein levels over the mitochondrial mass revealed an increased SDHC/mitochondrial mass ratio in GATA-1_{FL} cells with respect to GATA-1_S cells. According to the role of SDHC

overexpression in promoting mitochondrial superoxide production, this data well correlates with the higher mitochondrial concentration of superoxide in GATA-1_{FL} cells with respect to GATA-1_S cells.

Therefore, considering these findings, it is possible to speculate on a role for GATA-1_S in the regulation of complex II activity and its potential pro-leukemic significance. Two isoforms of SDHC are produced by alternative splicing mechanisms: Δ3 alternative splicing variant (Δ3 ASV), which lacks exon 3 encoding the oxidoreductase activity region and Δ5 ASV, defective of the exon 5 encoding the binding region for the heme-b 560. Δ3 and Δ5 ASVs are both ubiquitously expressed even at almost two-fold lower level than the full-length mRNA (T. Ishii et al. 2005; Satoh et al. 2015). Conversely, these isoforms are over-expressed in different tumor lines, such as HCT-15 colorectal adenocarcinoma cells, where the increase in the expression of SDHC Δ3 and Δ5 ASVs was found associated with reduced SDH activity and increased production of O_2^- (Andrews et al. 2018; Slane et al. 2006). Therefore, based on these observations, the expression levels of these two SDHC ASVs were evaluated in K562 over-expressing GATA-1 isoforms revealing that upregulation of SDHC in GATA-1_S cells was accompanied by a prevalence of the SDHC Δ5 ASV transcript compared to GATA-1_{FL} cells. In addition, expression levels of SDHC Δ5 in GATA-1_S cells inversely correlated with the SQR activity of complex II. Interestingly, these results are in agreement with the dominant-negative inhibition so far reported for the SDHC Δ5 isoform on its full-length variant (Satoh et al. 2015) as well as with the evidence illustrated in this thesis regarding increased levels of O_2^- associated with GATA-1_S over-expression (Riccio et al. 2019). Conversely, GATA-1_S knock-down was accompanied by reduced expression of SDHC, particularly with regard to its Δ5 ASV variant. As a whole, these findings strongly corroborate the hypothesis that the leukemogenic potential of GATA-1_S can be related to complex II dysfunction.

In line with these results, GATA-1 isoforms were also found to differently influence mitochondria metabolism, suggesting that, at least in part, the larger mitochondrial network in GATA-1_S cells is hindered to efficiently contribute to the oxidative metabolism due to molecular mechanisms limiting OXPHOS. These findings are in agreement with a large body of literature indicating that, compared with their normal counterpart, AML cells display a larger mitochondrial network without increased respiratory chain activity alongside a lower spare reserve capacity that makes them more susceptible to oxidative stress (Al Ageeli 2020; Panuzzo et al. 2020; Trombetti, Cesaro, et al. 2021; Panina et al. 2019; Nelson et al. 2021; Marchetti et al. 2020). In this regard, it is also to be noted that, as previously discussed, GATA-1_S over-expression is associated with enhanced rates of mitochondrial fusion. This process helps mitigate stress conditions by mixing the content of partially damaged mitochondria, thus reducing pro-apoptotic signals, and increasing cell survival. Therefore, collectively, these results are clearly indicative of a mechanism through which GATA-1_S could exert a pro-leukemic role.

In addition, the higher ECAR values detected in GATA-1_S cells correlates with an enhanced glycolytic flux that invokes the pseudo-hypoxic phenotype occurring when the HIF-1α pathway is constitutively activated, regardless of oxygen levels, a condition that, as above mentioned, characterizes cancer cells and can be driven by loss of complex II activity (Laukka et al. 2016; Ghanbari Movahed et al. 2019). Based on these observations, HIF-1α levels were evaluated in these cells revealing high HIF-1α expression levels only in cells over-expressing GATA-1_S. Although more studies are required to better clarify the regulatory network involving GATA-1 and HIF-1α in normal and aberrant hematopoiesis, these finding shed new light on the molecular mechanisms leading to metabolic rewiring in leukemia cells.

Literature data also indicate that over-expression of defective SDHC variants is associated with increased production of O_2^-, enhanced oxidative stress and reinforced antioxidant defense systems that contribute to limit oxidative damage caused by excessive ROS production (T. Ishii et al. 2005; Slane et al. 2006; N. Ishii, Ishii, and Hartman 2006). Interestingly, these findings resemble what had previously seen in GATA-1_S cells showing elevated mitochondrial O_2^- levels along with enhanced antioxidant defenses due to increased levels of GSH and SOD1. Further light on the leukemogenic potential of GATA-1_S is provided by the altered redox state in these cells that can be associated with defective SDHC expression, impaired complex II activity, and reduced OXPHOS efficiency. Moreover, another aspect that needs to be considered is the relevant contribution of dysregulated alternative splicing mechanisms generating GATA-1_S and SDHC ASVs that, in our hands, show an high leukemogenic potential. In this context it is interesting to note that, whereas alternative splicing variants extensively contributes to the regulation of organ development and cell differentiation programs, disruption of the splicing machinery is associated with human diseases and can contribute to oncogenesis and development of drug resistance in cancer cells (Cunningham et al. 2013). Furthermore, as largely reviewed, maintenance of normal isoform ratios is crucial to control lineage commitment and progenitor maturation in normal hematopoiesis (Shimizu and Yamamoto 2012; Halsey et al. 2010; Grech et al. 2014).

Therefore, although more research is needed to explore the role of GATA-1 N-terminal transactivation domain to modulate gene expression, including those genes involved in the regulation of cellular metabolism, it is arguable that GATA-1 could also been involved in regulation of gene expression at the post-transcriptional level by affecting the alternative splicing machinery, as suggested by the aberrant expression of SDHC ASVs associated with GATA-1S over-expression.

Finally, with the aim to explore the potential clinical impact of our findings, GATA-1 isoforms and SDHC levels were evaluated in bone marrow samples from a patient with AML at different stages of the disease. Results showed a dramatic increase of GATA-1_S levels at diagnosis that were reduced after chemotherapy and normalized at remission, thus confirming the data obtained in K562 cells as indicative of a pro-leukemic role of GATA-1_S with respect to its

full- length counterpart. Also, consistently with data from K562 cells, a similar trend was found for SDHC isoforms levels. Therefore, although more AML samples are required to corroborate the clinical relevance of these findings, these preliminary results are in full agreement with our experimental model, therefore providing support to the potential clinical implications of this study and further highlighting the key role played by mitochondria in these pathogenic processes.

6.CONCLUSION

The mitochondrial network acts as a central hub that directly or indirectly controls many cellular processes including proliferation, ATP synthesis and cell death through the complex integration of metabolic, bioenergetic and redox signals. The metabolic reprogramming of these organelles contributes to the development and progression of leukemia.

This study allowed to clarify the different effects exerted by GATA-1 isoforms in myeloid precursors and in mitochondrial remodeling. The putative pathophysiological effects exerted by GATA-1_S in the leukemogenic process were initially examined through the modulation, production, and compartmentalization of ROS. Results showed that GATA-1_S expression triggers increased mitochondrial superoxide anion production and increased mitochondrial mass following mitochondrial remodeling induced by fusion processes as a mechanism to mitigate mitochondrial stress conditions.

A link between the expression levels of the GATA-1 isoforms and the levels of SDHC ASVs was found that leads to inhibition of respiratory chain complex II activity and reduced efficiency of oxidative phosphorylation, thus shedding novel light on the molecular mechanisms through which GATA-1_S could contribute to the onset and development of leukemia.

Although many questions remain to be addressed, this study highlights a hitherto unexplored goal for ROS intervention. In fact, by contributing to a better knowledge of the sources and species of ROS generated in myeloid cells to escape apoptosis and to promote cell proliferation and leukemogenesis, this study could pave the way to new and more effective ROS-based therapies in myeloid leukemia, particularly in drug-resistant diseases.

7. LIST OF PUBLICATIONS

- **Silvia Trombetti**, Raffaele Sessa, Rosa Catapano, Laura Rinaldi, Alessandra Lo Bianco, Antonio Feliciello, Paola Izzo, Michela Grosso, Exploring the leukemogenic potential of GATA-1S, the shorter isoform of GATA-1: novel insights into mechanisms hampering respiratory chain complex II activity and limiting oxidative phosphorylation efficiency, Antioxidants 2021,10, 1603. https://doi.org/10.3390/antiox10101603
- Raffaella Liccardo, Raffaele Sessa, **Silvia Trombetti**, Marina De Rosa, Paola Izzo, Michela Grosso, Francesca Duraturo, MiR-137 Targets the 3' Untranlated Region of MSH2: Potential Implications in Lynch Syndrome-Related Colorectal Cancer, Cancers 2021, 13, 4662. https://doi.org/10.3390/cancers13184662
- **Silvia Trombetti**, Elena Cesaro, Rosa Catapano, Raffaele Sessa, Alessandra Lo Bianco, Paola Izzo and Michela Grosso, Oxidative Stress and ROS-Mediated Signaling in Leukemia: Novel Promising Perspectives to Eradicate Chemoresistant Cells in Myeloid Leukemia Int. J. Mol. Sci. 2021, 22, 2470. https://doi.org/10.3390/ijms22052470
- Marianna De Martino, Raffaele Sessa, Maria Rosaria Storino, Mariarosaria Giuliano, **Silvia Trombetti**, Rosa Catapano, Alessandra Lo Bianco, Paola Izzo and Michela Grosso, Transcriptional Repressors of Fetal Globin Genes as Novel Therapeutic Targets in Beta-Thalassemia doi: 10.5772/intechopen.90762
- Patrizia Riccio, Raffaele Sessa, Sergio de Nicola, Fara Petruzziello, **Silvia Trombetti**, Giuseppe Menna, Giampiero Pepe, Pasquale Maddalena, Paola Izzo and Michela Grosso, GATA-1 isoforms differently contribute to the production and compartmentation of reactive oxygen species in the myeloid leukemia cell line K562, J Cell Physiology 2019 doi: 10.1002/jcp.2868

REFERENCES

Akan, Zafer, and Ayse Inhan Garip. 2013. "Antioxidants May Protect Cancer Cells from Apoptosis Signals and Enhance Cell Viability." *Asian Pacific Journal of Cancer Prevention* 14 (8): 4611–14. https://doi.org/10.7314/APJCP.2013.14.8.4611.

Al Ageeli, Essam. 2020. "Alterations of Mitochondria and Related Metabolic Pathways in Leukemia: A Narrative Review." *Saudi Journal of Medicine and Medical Sciences* 8 (1): 3. https://doi.org/10.4103/sjmms.sjmms_112_18.

Amar, Laurence, Karel Pacak, Olivier Steichen, Scott A. Akker, Simon J. B. Aylwin, Eric Baudin, Alexandre Buffet, et al. 2021. "International Consensus on Initial Screening and Follow-up of Asymptomatic SDHx Mutation Carriers." *Nature Reviews Endocrinology* 17 (7): 435–44. https://doi.org/10.1038/s41574-021-00492-3.

Andrews, Katrina A, David B Ascher, Douglas Eduardo Valente Pires, Daniel R Barnes, Lindsey Vialard, Ruth T Casey, Nicola Bradshaw, et al. 2018. "Tumour Risks and Genotype–Phenotype Correlations Associated with Germline Variants in Succinate Dehydrogenase Subunit Genes *SDHB* , *SDHC* and *SDHD*." *Journal of Medical Genetics* 55 (6): 384–94. https://doi.org/10.1136/jmedgenet-2017-105127.

Balduini, Carlo, Alessandro Pecci, Giuseppe Loffredo, Paola Izzo, Patrizia Noris, Michela Grosso, Gaetano Bergamaschi, et al. 2004. "Effects of the R216Q Mutation of GATA-1 on Erythropoiesis and Megakaryocytopoiesis." *Thrombosis and Haemostasis* 91 (01): 129–40. https://doi.org/10.1160/TH03-05-0290.

Bansal, Ankita, and M. Celeste Simon. 2018. "Glutathione Metabolism in Cancer Progression and Treatment Resistance." *Journal of Cell Biology* 217 (7): 2291–98. https://doi.org/10.1083/jcb.201804161.

Battisti, Vanessa, Liési D.K. Maders, Margarete D. Bagatini, Karen F. Santos, Rosélia M. Spanevello, Paula A. Maldonado, Alice O. Brulé, Maria do Carmo Araújo, Maria R.C. Schetinger, and Vera M. Morsch. 2008. "Measurement of Oxidative Stress and Antioxidant Status in Acute Lymphoblastic Leukemia Patients." *Clinical Biochemistry* 41 (7–8): 511–18. https://doi.org/10.1016/j.clinbiochem.2008.01.027.

Bell, Eric L., Tatyana A. Klimova, James Eisenbart, Carlos T. Moraes, Michael P. Murphy, G.R. Scott Budinger, and Navdeep S. Chandel. 2007. "The Qo Site of the Mitochondrial Complex III Is Required for the Transduction of Hypoxic Signaling via Reactive Oxygen Species Production." *Journal of Cell Biology* 177 (6): 1029–36. https://doi.org/10.1083/jcb.200609074.

Bezawork-Geleta, Ayenachew, Jakub Rohlena, Lanfeng Dong, Karel Pacak, and Jiri Neuzil. 2017. "Mitochondrial Complex II: At the Crossroads." *Trends in Biochemical Sciences* 42 (4): 312–25. https://doi.org/10.1016/j.tibs.2017.01.003.

Brand, Martin D. 2016. "Mitochondrial Generation of Superoxide and Hydrogen Peroxide as the Source of Mitochondrial Redox Signaling." *Free Radical*

Biology and Medicine 100 (November): 14–31. https://doi.org/10.1016/j.freeradbiomed.2016.04.001.

Bresnick, E. H., K. R. Katsumura, H.-Y. Lee, K. D. Johnson, and A. S. Perkins. 2012. "Master Regulatory GATA Transcription Factors: Mechanistic Principles and Emerging Links to Hematologic Malignancies." *Nucleic Acids Research* 40 (13): 5819–31. https://doi.org/10.1093/nar/gks281.

Brière, J.-J., J. Favier, V. El. Ghouzzi, F. Djouadi, P. Bénit, A. -P. Gimenez, and P. Rustin. 2005. "Succinate Dehydrogenase Deficiency in Human." *Cellular and Molecular Life Sciences* 62 (19–20): 2317–24. https://doi.org/10.1007/s00018-005-5237-6.

Brisdelli, Fabrizia, Cristina Coccia, Benedetta Cinque, Maria Grazia Cifone, and Argante Bozzi. 2007. "Induction of Apoptosis by Quercetin: Different Response of Human Chronic Myeloid (K562) and Acute Lymphoblastic (HSB-2) Leukemia Cells." *Molecular and Cellular Biochemistry* 296 (1–2): 137–49. https://doi.org/10.1007/s11010-006-9307-3.

Burda, P, P Laslo, and T Stopka. 2010. "The Role of PU.1 and GATA-1 Transcription Factors during Normal and Leukemogenic Hematopoiesis." *Leukemia* 24 (7): 1249–57. https://doi.org/10.1038/leu.2010.104.

Burda, Pavel, Jarmila Vargova, Nikola Curik, Cyril Salek, Giorgio Lucio Papadopoulos, John Strouboulis, and Tomas Stopka. 2016. "GATA-1 Inhibits PU.1 Gene via DNA and Histone H3K9 Methylation of Its Distal Enhancer in Erythroleukemia." Edited by Ken Mills. *PLOS ONE* 11 (3): e0152234. https://doi.org/10.1371/journal.pone.0152234.

Burda, Pavel, Jarmila Vargova, Nikola Curik, John Strouboulis, Giorgio Lucio Papadopoulos, Anna Jonasova, and Tomas Stopka. 2015. "Azacitidine Blocks GATA-1-Mediated Repression of the PU.1 Gene in Human Leukemic Cells." *Blood* 126 (23): 5220–5220. https://doi.org/10.1182/blood.V126.23.5220.5220.

Caldwell, John Timothy, Holly Edwards, Alan A. Dombkowski, Steven A. Buck, Larry H. Matherly, Yubin Ge, and Jeffrey W. Taub. 2013. "Overexpression of GATA1 Confers Resistance to Chemotherapy in Acute Megakaryocytic Leukemia." Edited by Yves St-Pierre. *PLoS ONE* 8 (7): e68601. https://doi.org/10.1371/journal.pone.0068601.

Cecchini, Gary. 2003. "Function and Structure of Complex II of the Respiratory Chain." *Annual Review of Biochemistry* 72 (1): 77–109. https://doi.org/10.1146/annurev.biochem.72.121801.161700.

Chen, Yu-Lin, and Wai-Ming Kan. 2015. "Down-Regulation of Superoxide Dismutase 1 by PMA Is Involved in Cell Fate Determination and Mediated via Protein Kinase D2 in Myeloid Leukemia Cells." *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1853 (10): 2662–75. https://doi.org/10.1016/j.bbamcr.2015.07.025.

Chlon, T. M., M. McNulty, B. Goldenson, A. Rosinski, and J. D. Crispino. 2015. "Global Transcriptome and Chromatin Occupancy Analysis Reveal the Short Isoform of GATA1 Is Deficient for Erythroid Specification and Gene Expression." *Haematologica* 100 (5): 575–84. https://doi.org/10.3324/haematol.2014.112714.

Ciarcia, Roberto, Danila d'Angelo, Carmen Pacilio, David Pagnini, Massimiliano Galdiero, Filomena Fiorito, Sara Damiano, et al. 2010. "Dysregulated Calcium Homeostasis and Oxidative Stress in Chronic Myeloid Leukemia (CML) Cells." *Journal of Cellular Physiology* 224 (2): 443–53. https://doi.org/10.1002/jcp.22140.

Ciccarone, Fabio, Luca Di Leo, and Maria Rosa Ciriolo. 2018. "Aberrations of the TCA Cycle in Cancer." In *Reference Module in Biomedical Sciences*, B9780128012383650000. Elsevier. https://doi.org/10.1016/B978-0-12-801238-3.65066-3.

Ciovacco, Wendy A., Wendy H. Raskind, and Melissa A. Kacena. 2008. "Human Phenotypes Associated with GATA-1 Mutations." *Gene* 427 (1–2): 1–6. https://doi.org/10.1016/j.gene.2008.09.018.

Cockfield, Jordan A., and Zachary T. Schafer. 2019. "Antioxidant Defenses: A Context-Specific Vulnerability of Cancer Cells." *Cancers* 11 (8): 1208. https://doi.org/10.3390/cancers11081208.

Cramer-Morales, Kimberly L, Collin D Heer, Kranti A Mapuskar, and Frederick E Domann. 2021. "Succinate Accumulation Links Mitochondrial MnSOD Depletion to Aberrant Nuclear DNA Methylation and Altered Cell Fate," 19.

Crispino, John D. 2005. "GATA1 in Normal and Malignant Hematopoiesis." *Seminars in Cell & Developmental Biology* 16 (1): 137–47. https://doi.org/10.1016/j.semcdb.2004.11.002.

Cunningham, Thomas J., Ilaria Palumbo, Michela Grosso, Nicholas Slater, and Colin G. Miles. 2013. "WT1 Regulates Murine Hematopoiesis via Maintenance of VEGF Isoform Ratio." *Blood* 122 (2): 188–92. https://doi.org/10.1182/blood-2012-11-466086.

Dalla Pozza, Elisa, Ilaria Dando, Raffaella Pacchiana, Elio Liboi, Maria Teresa Scupoli, Massimo Donadelli, and Marta Palmieri. 2020. "Regulation of Succinate Dehydrogenase and Role of Succinate in Cancer." *Seminars in Cell & Developmental Biology* 98 (February): 4–14. https://doi.org/10.1016/j.semcdb.2019.04.013.

De Sousa, Sunita M C, John Toubia, Tristan S E Hardy, Jinghua Feng, Paul Wang, Andreas W Schreiber, Joel Geoghegan, et al. 2020. "Aberrant Splicing of *SDHC* in Families With Unexplained Succinate Dehydrogenase-Deficient Paragangliomas." *Journal of the Endocrine Society* 4 (12): bvaa071. https://doi.org/10.1210/jendso/bvaa071.

Di Marcantonio, Daniela, Esteban Martinez, Simone Sidoli, Jessica Vadaketh, Margaret Nieborowska-Skorska, Anushk Gupta, Jake M. Meadows, et al. 2018. "Protein Kinase C Epsilon Is a Key Regulator of Mitochondrial Redox Homeostasis in Acute Myeloid Leukemia." *Clinical Cancer Research* 24 (3): 608–18. https://doi.org/10.1158/1078-0432.CCR-17-2684.

Doré, Louis C., and John D. Crispino. 2011. "Transcription Factor Networks in Erythroid Cell and Megakaryocyte Development." *Blood* 118 (2): 231–39. https://doi.org/10.1182/blood-2011-04-285981.

Du, Jian, Monika J. Stankiewicz, Yang Liu, Qing Xi, Jonathan E. Schmitz, Julie A. Lekstrom-Himes, and Steven J. Ackerman. 2002. "Novel Combinatorial

Interactions of GATA-1, PU.1, and C/EBPε Isoforms Regulate Transcription of the Gene Encoding Eosinophil Granule Major Basic Protein." *Journal of Biological Chemistry* 277 (45): 43481–94. https://doi.org/10.1074/jbc.M204777200.

Er, Tze-Kiong, Shih-Meng Tsai, Szu-Hsien Wu, Wen Chiang, Hsien-Cheng Lin, Sheng-Fung Lin, Szu-Hua Wu, Li-Yu Tsai, and Tsan-Zon Liu. 2007. "Antioxidant Status and Superoxide Anion Radical Generation in Acute Myeloid Leukemia." *Clinical Biochemistry* 40 (13–14): 1015–19. https://doi.org/10.1016/j.clinbiochem.2007.05.013.

Farge, Thomas, Estelle Saland, Fabienne de Toni, Nesrine Aroua, Mohsen Hosseini, Robin Perry, Claudie Bosc, et al. 2017. "Chemotherapy-Resistant Human Acute Myeloid Leukemia Cells Are Not Enriched for Leukemic Stem Cells but Require Oxidative Metabolism." *Cancer Discovery* 7 (7): 716–35. https://doi.org/10.1158/2159-8290.CD-16-0441.

Ferraresi, Roberta, Leonarda Troiano, Erika Roat, Enrico Lugli, Elisa Nemes, Milena Nasi, Marcello Pinti, Maria I. Garcia Fernandez, Edwin L. Cooper, and Andrea Cossarizza. 2005. "Essential Requirement of Reduced Glutathione (GSH) for the Anti-Oxidant Effect of the Flavonoid Quercetin." *Free Radical Research* 39 (11): 1249–58. https://doi.org/10.1080/10715760500306935.

Ferreira, Rita, Kinuko Ohneda, Masayuki Yamamoto, and Sjaak Philipsen. 2005. "GATA1 Function, a Paradigm for Transcription Factors in Hematopoiesis." *Molecular and Cellular Biology* 25 (4): 1215–27. https://doi.org/10.1128/MCB.25.4.1215-1227.2005.

Fonseca, Tiago Branco, Ángela Sánchez-Guerrero, Ira Milosevic, and Nuno Raimundo. 2019. "Mitochondrial Fission Requires DRP1 but Not Dynamins." *Nature* 570 (7761): E34–42. https://doi.org/10.1038/s41586-019-1296-y.

Franco, Leticia V. R., Luca Bremner, and Mario H. Barros. 2020. "Human Mitochondrial Pathologies of the Respiratory Chain and ATP Synthase: Contributions from Studies of Saccharomyces Cerevisiae." *Life* 10 (11): 304. https://doi.org/10.3390/life10110304.

Fulkerson, Patricia C. 2017. "Transcription Factors in Eosinophil Development and As Therapeutic Targets." *Frontiers in Medicine* 4 (July): 115. https://doi.org/10.3389/fmed.2017.00115.

Gao, Juehua, Yi-Hua Chen, and LoAnn C. Peterson. 2015. "GATA Family Transcriptional Factors: Emerging Suspects in Hematologic Disorders." *Experimental Hematology & Oncology* 4 (1): 28. https://doi.org/10.1186/s40164-015-0024-z.

Garnett, Catherine, David Cruz Hernandez, and Paresh Vyas. 2020. "GATA1 and Cooperating Mutations in Myeloid Leukaemia of Down Syndrome." *IUBMB Life* 72 (1): 119–30. https://doi.org/10.1002/iub.2197.

Ghanbari Movahed, Zahra, Mohsen Rastegari-Pouyani, Mohammad hossein Mohammadi, and Kamran Mansouri. 2019. "Cancer Cells Change Their Glucose Metabolism to Overcome Increased ROS: One Step from Cancer Cell to Cancer Stem Cell?" *Biomedicine & Pharmacotherapy* 112 (April): 108690. https://doi.org/10.1016/j.biopha.2019.108690.

Girerd, Sandrine, Lucie Tosca, Olivier Herault, Christine Vignon, Denis Biard, Djamel Aggoune, Fatima Dkhissi, et al. 2018. "Superoxide Dismutase 2 (SOD2) Contributes to Genetic Stability of Native and T315I-Mutated BCR-ABL Expressing Leukemic Cells." *Biochemical and Biophysical Research Communications* 498 (4): 715–22. https://doi.org/10.1016/j.bbrc.2018.03.023.

Gonçalves, Rui Pedro, Nikolay Buzhynskyy, Valérie Prima, James N. Sturgis, and Simon Scheuring. 2007. "Supramolecular Assembly of VDAC in Native Mitochondrial Outer Membranes." *Journal of Molecular Biology* 369 (2): 413–18. https://doi.org/10.1016/j.jmb.2007.03.063.

Grech, Godfrey, Joel Pollacco, Mark Portelli, Keith Sacco, Shawn Baldacchino, Justine Grixti, and Christian Saliba. 2014. "Expression of Different Functional Isoforms in Haematopoiesis." *International Journal of Hematology* 99 (1): 4–11. https://doi.org/10.1007/s12185-013-1477-7.

Gregory, Gregory D, Annarita Miccio, Alexey Bersenev, Yuhuan Wang, Wei Hong, Zhe Zhang, Mortimer Poncz, Wei Tong, and Gerd A Blobel. 2010. "FOG1 Requires NuRD to Promote Hematopoiesis and Maintain Lineage FIdelity within the Megakaryocytic-Erythroid Compartment" 115 (11): 11.

Grimm, Stefan. 2013. "Respiratory Chain Complex II as General Sensor for Apoptosis." *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1827 (5): 565–72. https://doi.org/10.1016/j.bbabio.2012.09.009.

Gruber, Tanja A., and James R. Downing. 2015. "The Biology of Pediatric Acute Megakaryoblastic Leukemia." *Blood* 126 (8): 943–49. https://doi.org/10.1182/blood-2015-05-567859.

Guo, Runyu, Shuai Zong, Meng Wu, Jinke Gu, and Maojun Yang. 2017. "Architecture of Human Mitochondrial Respiratory Megacomplex I2III2IV2." *Cell* 170 (6): 1247-1257.e12. https://doi.org/10.1016/j.cell.2017.07.050.

Gurbuxani, Sandeep, Paresh Vyas, and John D. Crispino. 2004. "Recent Insights into the Mechanisms of Myeloid Leukemogenesis in Down Syndrome." *Blood* 103 (2): 399–406. https://doi.org/10.1182/blood-2003-05-1556.

Gutiérrez, Laura, Noemí Caballero, Luis Fernández-Calleja, Elena Karkoulia, and John Strouboulis. 2020. "Regulation of GATA1 Levels in Erythropoiesis." *IUBMB Life* 72 (1): 89–105. https://doi.org/10.1002/iub.2192.

Guzzo, Giulia, Marco Sciacovelli, Paolo Bernardi, and Andrea Rasola. 2014. "Inhibition of Succinate Dehydrogenase by the Mitochondrial Chaperone TRAP1 Has Anti-Oxidant and Anti-Apoptotic Effects on Tumor Cells." *Oncotarget* 5 (23): 11897–908. https://doi.org/10.18632/oncotarget.2472.

Halsey, Christina, Marie Docherty, Mhairi McNeill, Derek Gilchrist, Michelle Le Brocq, Brenda Gibson, and Gerard Graham. 2012. "The GATA1s Isoform Is Normally Down-Regulated during Terminal Haematopoietic Differentiation and over-Expression Leads to Failure to Repress MYB, CCND2 and SKI during Erythroid Differentiation of K562 Cells." *Journal of Hematology & Oncology* 5 (1): 45. https://doi.org/10.1186/1756-8722-5-45.

Halsey, Christina, Oliver Tunstall, Brenda Gibson, Irene Roberts, and Gerard Graham. 2010. "Role of GATA-1s in Early Hematopoiesis and Differences between Alternative Splicing in Human and Murine GATA-1." *Blood* 115 (16): 3415–

16. https://doi.org/10.1182/blood-2010-02-267930.
Harigae, Hideo. 2006. “GATA Transcription Factors and Hematological Diseases.” *The Tohoku Journal of Experimental Medicine* 210 (1): 1–9. https://doi.org/10.1620/tjem.210.1.
Hasegawa, Atsushi, and Ritsuko Shimizu. 2017. “GATA1 Activity Governed by Configurations of Cis-Acting Elements.” *Frontiers in Oncology* 6 (January). https://doi.org/10.3389/fonc.2016.00269.
He, Long, Ting He, Shabnam Farrar, Linbao Ji, Tianyi Liu, and Xi Ma. 2017. “Antioxidants Maintain Cellular Redox Homeostasis by Elimination of Reactive Oxygen Species.” *Cellular Physiology and Biochemistry* 44 (2): 532–53. https://doi.org/10.1159/000485089.
Hole, Paul Spencer, Richard Lawrence Darley, and Alex Tonks. 2011. “Do Reactive Oxygen Species Play a Role in Myeloid Leukemias?” *Blood* 117 (22): 5816–26. https://doi.org/10.1182/blood-2011-01-326025.
Holmström, Kira M., and Toren Finkel. 2014. “Cellular Mechanisms and Physiological Consequences of Redox-Dependent Signalling.” *Nature Reviews Molecular Cell Biology* 15 (6): 411–21. https://doi.org/10.1038/nrm3801.
Huang, Peng, Li Feng, Elizabeth A. Oldham, Michael J. Keating, and William Plunkett. 2000. “Superoxide Dismutase as a Target for the Selective Killing of Cancer Cells.” *Nature* 407 (6802): 390–95. https://doi.org/10.1038/35030140.
Irwin, Mary E., Nilsa Rivera-Del Valle, and Joya Chandra. 2013. “Redox Control of Leukemia: From Molecular Mechanisms to Therapeutic Opportunities.” *Antioxidants & Redox Signaling* 18 (11): 1349–83. https://doi.org/10.1089/ars.2011.4258.
Ishii, Naoaki, Takamasa Ishii, and Philip S. Hartman. 2006. “The Role of the Electron Transport Gene SDHC on Lifespan and Cancer.” *Experimental Gerontology* 41 (10): 952–56. https://doi.org/10.1016/j.exger.2006.06.037.
Ishii, Takamasa, Kayo Yasuda, Akira Akatsuka, Okio Hino, Philip S Hartman, and Naoaki Ishii. 2005. “A Mutation in the SDHC Gene of Complex II Increases Oxidative Stress, Resulting in Apoptosis and Tumorigenesis.” *Cancer Res*, 8.
Iwata, So, Joong W. Lee, Kengo Okada, John Kyongwon Lee, Momi Iwata, Bjarne Rasmussen, Thomas A. Link, S. Ramaswamy, and Bing K. Jap. 1998. “Complete Structure of the 11-Subunit Bovine Mitochondrial Cytochrome Bc 1 Complex.” *Science* 281 (5373): 64–71. https://doi.org/10.1126/science.281.5373.64.
Jäger, R, H Gisslinger, F Passamonti, E Rumi, T Berg, B Gisslinger, D Pietra, et al. 2010. “Deletions of the Transcription Factor Ikaros in Myeloproliferative Neoplasms.” *Leukemia* 24 (7): 1290–98. https://doi.org/10.1038/leu.2010.99.
Ježek, Jan, Katrina Cooper, and Randy Strich. 2018. “Reactive Oxygen Species and Mitochondrial Dynamics: The Yin and Yang of Mitochondrial Dysfunction and Cancer Progression.” *Antioxidants* 7 (1): 13. https://doi.org/10.3390/antiox7010013.
Jitschin, Regina, Andreas D. Hofmann, Heiko Bruns, Andreas Gießl, Juliane Bricks, Jana Berger, Domenica Saul, Michael J. Eckart, Andreas Mackensen, and Dimitrios Mougiakakos. 2014. “Mitochondrial Metabolism Contributes to

Oxidative Stress and Reveals Therapeutic Targets in Chronic Lymphocytic Leukemia." *Blood* 123 (17): 2663–72. https://doi.org/10.1182/blood-2013-10-532200.

Jonckheere, An I., G. Herma Renkema, Maaike Bras, Lambert P. van den Heuvel, Alexander Hoischen, Christian Gilissen, Sander B. Nabuurs, et al. 2013. "A Complex V ATP5A1 Defect Causes Fatal Neonatal Mitochondrial Encephalopathy." *Brain* 136 (5): 1544–54. https://doi.org/10.1093/brain/awt086.

Kacena, Melissa A., Caren M. Gundberg, William J. Kacena, William J. Landis, Adele L. Boskey, Mary L. Bouxsein, and Mark C. Horowitz. 2013. "The Effects of GATA-1 and NF-E2 Deficiency on Bone Biomechanical, Biochemical, and Mineral Properties." *Journal of Cellular Physiology* 228 (7): 1594–1600. https://doi.org/10.1002/jcp.24322.

Kaneko, Hiroshi, Eri Kobayashi, Masayuki Yamamoto, and Ritsuko Shimizu. 2012. "N- and C-Terminal Transactivation Domains of GATA1 Protein Coordinate Hematopoietic Program." *Journal of Biological Chemistry* 287 (25): 21439–49. https://doi.org/10.1074/jbc.M112.370437.

Kaweme, Natasha Mupeta, Shu Zhou, Geoffrey Joseph Changwe, and Fuling Zhou. 2020. "The Significant Role of Redox System in Myeloid Leukemia: From Pathogenesis to Therapeutic Applications." *Biomarker Research* 8 (1): 63. https://doi.org/10.1186/s40364-020-00242-z.

Khan, Irum, Sebastien Malinge, and John Crispino. 2011. "Myeloid Leukemia in Down Syndrome." *Critical Reviews™ in Oncogenesis* 16 (1–2): 25–36. https://doi.org/10.1615/CritRevOncog.v16.i1-2.40.

Kirschberg, Matthias, Sandra Heuser, Gian Paolo Marcuzzi, Martin Hufbauer, Jens Michael Seeger, Anamaria Đukić, Vjekoslav Tomaić, et al. 2020. "ATP Synthase Modulation Leads to an Increase of Spare Respiratory Capacity in HPV Associated Cancers." *Scientific Reports* 10 (1): 17339. https://doi.org/10.1038/s41598-020-74311-6.

Kirtonia, Anuradha, Gautam Sethi, and Manoj Garg. 2020. "The Multifaceted Role of Reactive Oxygen Species in Tumorigenesis." *Cellular and Molecular Life Sciences* 77 (22): 4459–83. https://doi.org/10.1007/s00018-020-03536-5.

Kobayashi, M., and M. Yamamoto. 2007. "Regulation of GATA1 Gene Expression." *Journal of Biochemistry* 142 (1): 1–10. https://doi.org/10.1093/jb/mvm122.

Kocabas, Fatih, Li Xie, Jingjing Xie, Zhuo Yu, Ralph J. DeBerardinis, Wataru Kimura, SuWannee Thet, et al. 2015. "Hypoxic Metabolism in Human Hematopoietic Stem Cells." *Cell & Bioscience* 5 (1): 39. https://doi.org/10.1186/s13578-015-0020-3.

Kumar, C. C. 2011. "Genetic Abnormalities and Challenges in the Treatment of Acute Myeloid Leukemia." *Genes & Cancer* 2 (2): 95–107. https://doi.org/10.1177/1947601911408076.

Lagadic-Gossmann, D, L Huc, and V Lecureur. 2004. "Alterations of Intracellular PH Homeostasis in Apoptosis: Origins and Roles." *Cell Death & Differentiation* 11 (9): 953–61. https://doi.org/10.1038/sj.cdd.4401466.

Laukka, Tuomas, Christopher J. Mariani, Tuukka Ihantola, John Z. Cao, Juho

Hokkanen, William G. Kaelin, Lucy A. Godley, and Peppi Koivunen. 2016. "Fumarate and Succinate Regulate Expression of Hypoxia-Inducible Genes via TET Enzymes." *Journal of Biological Chemistry* 291 (8): 4256–65. https://doi.org/10.1074/jbc.M115.688762.

Lee, Michelle H., Rena G. Lapidus, Dana Ferraris, and Ashkan Emadi. 2019. "Analysis of the Mechanisms of Action of Naphthoquinone-Based Anti-Acute Myeloid Leukemia Chemotherapeutics." *Molecules* 24 (17): 3121. https://doi.org/10.3390/molecules24173121.

Lemarie, Anthony, and Stefan Grimm. 2009. "Mutations in the Heme B-Binding Residue of SDHC Inhibit Assembly of Respiratory Chain Complex II in Mammalian Cells." *Mitochondrion* 9 (4): 254–60. https://doi.org/10.1016/j.mito.2009.03.004.

Lentjes, Marjolein HFM, Hanneke EC Niessen, Yoshimitsu Akiyama, Adriaan P de Bruïne, Veerle Melotte, and Manon van Engeland. 2016. "The Emerging Role of GATA Transcription Factors in Development and Disease." *Expert Reviews in Molecular Medicine* 18: e3. https://doi.org/10.1017/erm.2016.2.

Levine, Arthur S., Luxi Sun, Rong Tan, Ying Gao, Lu Yang, Hao Chen, Yaqun Teng, and Li Lan. 2017. "The Oxidative DNA Damage Response: A Review of Research Undertaken with Tsinghua and Xiangya Students at the University of Pittsburgh." *Science China Life Sciences* 60 (10): 1077–80. https://doi.org/10.1007/s11427-017-9184-6.

Ling, Te, and John D. Crispino. 2020. "GATA1 Mutations in Red Cell Disorders." *IUBMB Life* 72 (1): 106–18. https://doi.org/10.1002/iub.2177.

Malinge, Sébastien, Clarisse Thiollier, Timothy M. Chlon, Louis C. Doré, Lauren Diebold, Olivier Bluteau, Vinciane Mabialah, et al. 2013. "Ikaros Inhibits Megakaryopoiesis through Functional Interaction with GATA-1 and NOTCH Signaling." *Blood* 121 (13): 2440–51. https://doi.org/10.1182/blood-2012-08-450627.

Mancini, Elena, Alejandra Sanjuan-Pla, Luisa Luciani, Susan Moore, Amit Grover, Agnes Zay, Kasper D Rasmussen, et al. 2012. "FOG-1 and GATA-1 Act Sequentially to Specify Definitive Megakaryocytic and Erythroid Progenitors: FOG-1 and GATA-1 in Lineage Commitment." *The EMBO Journal* 31 (2): 351–65. https://doi.org/10.1038/emboj.2011.390.

Marchetti, Philippe, Quentin Fovez, Nicolas Germain, Raeeka Khamari, and Jérôme Kluza. 2020. "Mitochondrial Spare Respiratory Capacity: Mechanisms, Regulation, and Significance in Non-transformed and Cancer Cells." *The FASEB Journal* 34 (10): 13106–24. https://doi.org/10.1096/fj.202000767R.

Masuda, Akio, Katsunori Hashimoto, Toyoharu Yokoi, Takeshi Doi, Tatsuhiko Kodama, Hiroaki Kume, Kinji Ohno, and Tetsuya Matsuguchi. 2007. "Essential Role of GATA Transcriptional Factors in the Activation of Mast Cells." *The Journal of Immunology* 178 (1): 360–68. https://doi.org/10.4049/jimmunol.178.1.360.

Mattes, Katharina, Edo Vellenga, and Hein Schepers. 2019. "Differential Redox-Regulation and Mitochondrial Dynamics in Normal and Leukemic Hematopoietic Stem Cells: A Potential Window for Leukemia Therapy."

Critical Reviews in Oncology/Hematology 144 (December): 102814. https://doi.org/10.1016/j.critrevonc.2019.102814.

Migliaccio, Anna Rita, Rosa Alba Rana, Massimo Sanchez, Rodolfo Lorenzini, Lucia Centurione, Lucia Bianchi, Alessandro Maria Vannucchi, Giovanni Migliaccio, and Stuart H. Orkin. 2003. "GATA-1 as a Regulator of Mast Cell Differentiation Revealed by the Phenotype of the GATA-1low Mouse Mutant." *Journal of Experimental Medicine* 197 (3): 281–96. https://doi.org/10.1084/jem.20021149.

Moldogazieva, N. T., I. M. Mokhosoev, N. B. Feldman, and S. V. Lutsenko. 2018. "ROS and RNS Signalling: Adaptive Redox Switches through Oxidative/Nitrosative Protein Modifications." *Free Radical Research* 52 (5): 507–43. https://doi.org/10.1080/10715762.2018.1457217.

Moosavi, Behrooz, Xiao-lei Zhu, Wen-Chao Yang, and Guang-Fu Yang. 2020. "Genetic, Epigenetic and Biochemical Regulation of Succinate Dehydrogenase Function." *Biological Chemistry* 401 (3): 319–30. https://doi.org/10.1515/hsz-2019-0264.

Morceau, Franck, Michael Schnekenburger, Mario Dicato, and Marc Diederich. 2004. "GATA-1: Friends, Brothers, and Coworkers." *Annals of the New York Academy of Sciences* 1030 (1): 537–54. https://doi.org/10.1196/annals.1329.064.

Moriguchi, Takashi, and Masayuki Yamamoto. 2014. "A Regulatory Network Governing Gata1 and Gata2 Gene Transcription Orchestrates Erythroid Lineage Differentiation." *International Journal of Hematology* 100 (5): 417–24. https://doi.org/10.1007/s12185-014-1568-0.

Muntean, Andrew G., and John D. Crispino. 2005. "Differential Requirements for the Activation Domain and FOG-Interaction Surface of GATA-1 in Megakaryocyte Gene Expression and Development." *Blood* 106 (4): 1223–31. https://doi.org/10.1182/blood-2005-02-0551.

Nelson, Margaret AM, Kelsey L McLaughlin, James T Hagen, Hannah S Coalson, Cameron Schmidt, Miki Kassai, Kimberly A Kew, et al. 2021. "Intrinsic OXPHOS Limitations Underlie Cellular Bioenergetics in Leukemia." *ELife* 10 (June): e63104. https://doi.org/10.7554/eLife.63104.

Ni, Hong-Min, Jessica A. Williams, and Wen-Xing Ding. 2015. "Mitochondrial Dynamics and Mitochondrial Quality Control." *Redox Biology* 4 (April): 6–13. https://doi.org/10.1016/j.redox.2014.11.006.

Nishiura, Tetsuo, Keiichiro Suzuki, Tetsuo Kawaguchi, Hirohisa Nakao, Naohisa Kawamura, Masako Taniguchi, Yoshio Kanayama, Takeshi Yonezawa, Susumu Iizuka, and Naoyuki Taniguchi. 1992. "Elevated Serum Manganese Superoxide Dismutase in Acute Leukemias." *Cancer Letters* 62 (3): 211–15. https://doi.org/10.1016/0304-3835(92)90098-G.

Ohneda, Kinuko, Takashi Moriguchi, Shin'ya Ohmori, Yasushi Ishijima, Hironori Satoh, Sjaak Philipsen, and Masayuki Yamamoto. 2014. "Transcription Factor GATA1 Is Dispensable for Mast Cell Differentiation in Adult Mice." *Molecular and Cellular Biology* 34 (10): 1812–26. https://doi.org/10.1128/MCB.01524-13.

Pani, Giovambattista, Renata Colavitti, Barbara Bedogni, Salvatore Fusco, Daniela Ferraro, Silvia Borrello, and Tommaso Galeotti. 2004. "Mitochondrial Superoxide Dismutase: A Promising Target for New Anticancer Therapies." *Current Medicinal Chemistry* 11 (10): 1299–1308. https://doi.org/10.2174/0929867043365297.

Panina, Svetlana B., Natalia Baran, Fabio H. Brasil da Costa, Marina Konopleva, and Natalia V. Kirienko. 2019. "A Mechanism for Increased Sensitivity of Acute Myeloid Leukemia to Mitotoxic Drugs." *Cell Death & Disease* 10 (8): 617. https://doi.org/10.1038/s41419-019-1851-3.

Panuzzo, Cristina, Aleksandar Jovanovski, Barbara Pergolizzi, Lucrezia Pironi, Serena Stanga, Carmen Fava, and Daniela Cilloni. 2020. "Mitochondria: A Galaxy in the Hematopoietic and Leukemic Stem Cell Universe." *International Journal of Molecular Sciences* 21 (11): 3928. https://doi.org/10.3390/ijms21113928.

Paul, Franziska, Ya'ara Arkin, Amir Giladi, Diego Adhemar Jaitin, Ephraim Kenigsberg, Hadas Keren-Shaul, Deborah Winter, et al. 2016. "Transcriptional Heterogeneity and Lineage Commitment in Myeloid Progenitors." *Cell* 164 (1–2): 325. https://doi.org/10.1016/j.cell.2015.12.046.

Petruzziello, F., R. Sessa, E.D. Giovannone, M. Catania, R. Parasole, G. Menna, R. Cuccurullo, et al. 2013. "P-150 Prognostic Significance of GATA-1 and WT1 Levels in Pediatric Hematological Disorders." *Leukemia Research* 37 (May): S91–92. https://doi.org/10.1016/S0145-2126(13)70198-X.

Pizzino, Gabriele, Natasha Irrera, Mariapaola Cucinotta, Giovanni Pallio, Federica Mannino, Vincenzo Arcoraci, Francesco Squadrito, Domenica Altavilla, and Alessandra Bitto. 2017. "Oxidative Stress: Harms and Benefits for Human Health." *Oxidative Medicine and Cellular Longevity* 2017: 1–13. https://doi.org/10.1155/2017/8416763.

Prieto-Bermejo, Rodrigo, Marta Romo-González, Alejandro Pérez-Fernández, Carla Ijurko, and Ángel Hernández-Hernández. 2018. "Reactive Oxygen Species in Haematopoiesis: Leukaemic Cells Take a Walk on the Wild Side." *Journal of Experimental & Clinical Cancer Research* 37 (1): 125. https://doi.org/10.1186/s13046-018-0797-0.

Quinlan, Casey L., Adam L. Orr, Irina V. Perevoshchikova, Jason R. Treberg, Brian A. Ackrell, and Martin D. Brand. 2012. "Mitochondrial Complex II Can Generate Reactive Oxygen Species at High Rates in Both the Forward and Reverse Reactions." *Journal of Biological Chemistry* 287 (32): 27255–64. https://doi.org/10.1074/jbc.M112.374629.

Raimondi, Vittoria, Francesco Ciccarese, and Vincenzo Ciminale. 2020. "Oncogenic Pathways and the Electron Transport Chain: A DangeROS Liaison." *British Journal of Cancer* 122 (2): 168–81. https://doi.org/10.1038/s41416-019-0651-y.

Rampal, R., and M. E. Figueroa. 2016. "Wilms Tumor 1 Mutations in the Pathogenesis of Acute Myeloid Leukemia." *Haematologica* 101 (6): 672–79. https://doi.org/10.3324/haematol.2015.141796.

Rasheed, Mohamed Rizwan Haroon Al, and Gabor Tarjan. 2018. "Succinate Dehydrogenase Complex: An Updated Review." *Archives of Pathology &*

Laboratory Medicine 142 (12): 1564–70. https://doi.org/10.5858/arpa.2017-0285-RS.

Refinetti, Paulo, David Warren, Stephan Morgenthaler, and Per O. Ekstrøm. 2017. "Quantifying Mitochondrial DNA Copy Number Using Robust Regression to Interpret Real Time PCR Results." *BMC Research Notes* 10 (1): 593. https://doi.org/10.1186/s13104-017-2913-1.

Riccio, Patrizia, Raffaele Sessa, Sergio Nicola, Fara Petruzziello, Silvia Trombetti, Giuseppe Menna, Giampiero Pepe, Pasquale Maddalena, Paola Izzo, and Michela Grosso. 2019. "GATA-1 Isoforms Differently Contribute to the Production and Compartmentation of Reactive Oxygen Species in the Myeloid Leukemia Cell Line K562." *Journal of Cellular Physiology* 234 (11): 20829–46. https://doi.org/10.1002/jcp.28688.

Rinaldi, Laura, Maria Sepe, Rossella Delle Donne, Kristel Conte, Antonietta Arcella, Domenica Borzacchiello, Stefano Amente, et al. 2017. "Mitochondrial AKAP1 Supports MTOR Pathway and Tumor Growth." *Cell Death & Disease* 8 (6): e2842–e2842. https://doi.org/10.1038/cddis.2017.241.

Rosmarin, Alan G., Zhongfa Yang, and Karen K. Resendes. 2005. "Transcriptional Regulation in Myelopoiesis: Hematopoietic Fate Choice, Myeloid Differentiation, and Leukemogenesis." *Experimental Hematology* 33 (2): 131–43. https://doi.org/10.1016/j.exphem.2004.08.015.

Rutter, Jared, Dennis R. Winge, and Joshua D. Schiffman. 2010. "Succinate Dehydrogenase – Assembly, Regulation and Role in Human Disease." *Mitochondrion* 10 (4): 393–401. https://doi.org/10.1016/j.mito.2010.03.001.

Sakamuri, Siva S. V. P., Jared A. Sperling, Venkata N. Sure, Monica H. Dholakia, Nicholas R. Peterson, Ibolya Rutkai, Padmini S. Mahalingam, Ryosuke Satou, and Prasad V. G. Katakam. 2018. "Measurement of Respiratory Function in Isolated Cardiac Mitochondria Using Seahorse XFe24 Analyzer: Applications for Aging Research." *GeroScience* 40 (3): 347–56. https://doi.org/10.1007/s11357-018-0021-3.

Satoh, Nana, Chikako Yokoyama, Noriaki Itamura, Yoshiharu Miyajima-Nakano, and Hisashi Hisatomi. 2015. "Alternative Splicing Isoform in Succinate Dehydrogenase Complex, Subunit C Causes Downregulation of Succinate-Coenzyme Q Oxidoreductase Activity in Mitochondria." *Oncology Letters* 9 (1): 330–34. https://doi.org/10.3892/ol.2014.2699.

Schieber, Michael, and Navdeep S. Chandel. 2014. "ROS Function in Redox Signaling and Oxidative Stress." *Current Biology* 24 (10): R453–62. https://doi.org/10.1016/j.cub.2014.03.034.

Schulze, Harald, and Joseph Italiano, eds. 2016. *Molecular and Cellular Biology of Platelet Formation: Implications in Health and Disease*. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-319-39562-3.

Sena, Laura A., and Navdeep S. Chandel. 2012. "Physiological Roles of Mitochondrial Reactive Oxygen Species." *Molecular Cell* 48 (2): 158–67. https://doi.org/10.1016/j.molcel.2012.09.025.

Shadel, Gerald S., and Tamas L. Horvath. 2015. "Mitochondrial ROS Signaling in Organismal Homeostasis." *Cell* 163 (3): 560–69.

https://doi.org/10.1016/j.cell.2015.10.001.
Shimamoto, T, K Ohyashiki, Jh Ohyashiki, K Kawakubo, T Fujimura, H Iwama, S Nakazawa, and K Toyama. 1995. “The Expression Pattern of Erythrocyte/Megakaryocyte-Related Transcription Factors GATA-1 and the Stem Cell Leukemia Gene Correlates with Hematopoietic Differentiation and Is Associated with Outcome of Acute Myeloid Leukemia.” *Blood* 86 (8): 3173–80. https://doi.org/10.1182/blood.V86.8.3173.3173.
Shimizu, Ritsuko, and Masayuki Yamamoto. 2012. “Contribution of GATA1 Dysfunction to Multi-Step Leukemogenesis.” *Cancer Science* 103 (12): 2039–44. https://doi.org/10.1111/cas.12007.
Sierotzki, Helge, and Gabriel Scalliet. 2013. “A Review of Current Knowledge of Resistance Aspects for the Next-Generation Succinate Dehydrogenase Inhibitor Fungicides.” *Phytopathology*® 103 (9): 880–87. https://doi.org/10.1094/PHYTO-01-13-0009-RVW.
Sillar, Germon, DeIuliis, and Dun. 2019. “The Role of Reactive Oxygen Species in Acute Myeloid Leukaemia.” *International Journal of Molecular Sciences* 20 (23): 6003. https://doi.org/10.3390/ijms20236003.
Slane, Benjamin G., Nùkhet Aykin-Burns, Brian J. Smith, Amanda L. Kalen, Prabhat C. Goswami, Frederick E. Domann, and Douglas R. Spitz. 2006. “Mutation of Succinate Dehydrogenase Subunit C Results in Increased $O_2^{\cdot-}$, Oxidative Stress, and Genomic Instability.” *Cancer Research* 66 (15): 7615–20. https://doi.org/10.1158/0008-5472.CAN-06-0833.
Snezhkina, Anastasiya V., Anna V. Kudryavtseva, Olga L. Kardymon, Maria V. Savvateeva, Nataliya V. Melnikova, George S. Krasnov, and Alexey A. Dmitriev. 2019. “ROS Generation and Antioxidant Defense Systems in Normal and Malignant Cells.” *Oxidative Medicine and Cellular Longevity* 2019 (August): 1–17. https://doi.org/10.1155/2019/6175804.
Steinmeier, Johann, and Ralf Dringen. 2019. “Exposure of Cultured Astrocytes to Menadione Triggers Rapid Radical Formation, Glutathione Oxidation and Mrp1-Mediated Export of Glutathione Disulfide.” *Neurochemical Research* 44 (5): 1167–81. https://doi.org/10.1007/s11064-019-02760-1.
Stopka, Tomas, Derek F Amanatullah, Michael Papetti, and Arthur I Skoultchi. 2005. “PU.1 Inhibits the Erythroid Program by Binding to GATA-1 on DNA and Creating a Repressive Chromatin Structure.” *The EMBO Journal* 24 (21): 3712–23. https://doi.org/10.1038/sj.emboj.7600834.
Sun, Fei, Xia Huo, Yujia Zhai, Aojin Wang, Jianxing Xu, Dan Su, Mark Bartlam, and Zihe Rao. 2005. “Crystal Structure of Mitochondrial Respiratory Membrane Protein Complex II.” *Cell* 121 (7): 1043–57. https://doi.org/10.1016/j.cell.2005.05.025.
Suzuki, Mikiko, Maki Kobayashi-Osaki, Shuichi Tsutsumi, Xiaoqing Pan, Shin’ya Ohmori, Jun Takai, Takashi Moriguchi, et al. 2013. “GATA Factor Switching from GATA2 to GATA1 Contributes to Erythroid Differentiation.” *Genes to Cells* 18 (11): 921–33. https://doi.org/10.1111/gtc.12086.
Tremblay, Mathieu, Oraly Sanchez-Ferras, and Maxime Bouchard. 2018. “GATA Transcription Factors in Development and Disease.” *Development* 145 (20):

dev164384. https://doi.org/10.1242/dev.164384.
Tretter, Laszlo, Attila Patocs, and Christos Chinopoulos. 2016. “Succinate, an Intermediate in Metabolism, Signal Transduction, ROS, Hypoxia, and Tumorigenesis.” *Biochimica et Biophysica Acta (BBA) - Bioenergetics* 1857 (8): 1086–1101. https://doi.org/10.1016/j.bbabio.2016.03.012.
Trombetti, Silvia, Elena Cesaro, Rosa Catapano, Raffaele Sessa, Alessandra Lo Bianco, Paola Izzo, and Michela Grosso. 2021. “Oxidative Stress and ROS-Mediated Signaling in Leukemia: Novel Promising Perspectives to Eradicate Chemoresistant Cells in Myeloid Leukemia.” *International Journal of Molecular Sciences* 22 (5): 2470. https://doi.org/10.3390/ijms22052470.
Trombetti, Silvia, Raffaele Sessa, Rosa Catapano, Laura Rinaldi, Alessandra Lo Bianco, Antonio Feliciello, Paola Izzo, and Michela Grosso. 2021. “Exploring the Leukemogenic Potential of GATA-1S, the Shorter Isoform of GATA-1: Novel Insights into Mechanisms Hampering Respiratory Chain Complex II Activity and Limiting Oxidative Phosphorylation Efficiency.” *Antioxidants* 10 (10): 1603. https://doi.org/10.3390/antiox10101603.
Udensi, Udensi K, and Paul B Tchounwou. 2014. “Dual Effect of Oxidative Stress on Leukemia Cancer Induction and Treatment.” *Journal of Experimental & Clinical Cancer Research* 33 (1): 106. https://doi.org/10.1186/s13046-014-0106-5.
Vakoc, Christopher R., Danielle L. Letting, Nele Gheldof, Tomoyuki Sawado, M.A. Bender, Mark Groudine, Mitchell J. Weiss, Job Dekker, and Gerd A. Blobel. 2005. “Proximity among Distant Regulatory Elements at the β-Globin Locus Requires GATA-1 and FOG-1.” *Molecular Cell* 17 (3): 453–62. https://doi.org/10.1016/j.molcel.2004.12.028.
Wang, Yan-Hong, Xiang-Jiu Xu, Ling-Fang Zhang, and Hong-Ling Li. 2014. “Mimic of Manganese Superoxide Dismutase Induces Apoptosis in Human Acute Myeloid Leukemia Cells.” *Leukemia & Lymphoma* 55 (5): 1166–75. https://doi.org/10.3109/10428194.2013.825904.
Wang, Ying, Robyn Branicky, Alycia Noë, and Siegfried Hekimi. 2018. “Superoxide Dismutases: Dual Roles in Controlling ROS Damage and Regulating ROS Signaling.” *Journal of Cell Biology* 217 (6): 1915–28. https://doi.org/10.1083/jcb.201708007.
Wheaton, William W, Samuel E Weinberg, Robert B Hamanaka, Saul Soberanes, Lucas B Sullivan, Elena Anso, Andrea Glasauer, et al. 2014. “Metformin Inhibits Mitochondrial Complex I of Cancer Cells to Reduce Tumorigenesis.” *ELife* 3 (May): e02242. https://doi.org/10.7554/eLife.02242.
Xavier, Ana C., Holly Edwards, Alan A. Dombkowski, Tugce B. Balci, Jason N. Berman, Graham Dellaire, Chengzhi Xie, et al. 2011. “A Unique Role of GATA1s in Down Syndrome Acute Megakaryocytic Leukemia Biology and Therapy.” Edited by Eliana Saul Furquim Werneck Abdelhay. *PLoS ONE* 6 (11): e27486. https://doi.org/10.1371/journal.pone.0027486.
Xu, C, H Fu, L Gao, L Wang, W Wang, J Li, Y Li, et al. 2014. “BCR-ABL/GATA1/MiR-138 Mini Circuitry Contributes to the Leukemogenesis of Chronic Myeloid Leukemia.” *Oncogene* 33 (1): 44–54.

https://doi.org/10.1038/onc.2012.557.
Ye, Zhi-Wei, Jie Zhang, Danyelle M. Townsend, and Kenneth D. Tew. 2015. "Oxidative Stress, Redox Regulation and Diseases of Cellular Differentiation." *Biochimica et Biophysica Acta (BBA) - General Subjects* 1850 (8): 1607–21. https://doi.org/10.1016/j.bbagen.2014.11.010.
Youle, Richard J., and Alexander M. van der Bliek. 2012. "Mitochondrial Fission, Fusion, and Stress." *Science* 337 (6098): 1062–65. https://doi.org/10.1126/science.1219855.
Zhang, Bei-bei, Dao-gang Wang, Fen-fen Guo, and Chao Xuan. 2015. "Mitochondrial Membrane Potential and Reactive Oxygen Species in Cancer Stem Cells." *Familial Cancer* 14 (1): 19–23. https://doi.org/10.1007/s10689-014-9757-9.
Zhao, Ru-Zhou, Shuai Jiang, Lin Zhang, and Zhi-Bin Yu. 2019. "Mitochondrial Electron Transport Chain, ROS Generation and Uncoupling (Review)." *International Journal of Molecular Medicine*, May. https://doi.org/10.3892/ijmm.2019.4188.
Zorov, Dmitry B., Magdalena Juhaszova, and Steven J. Sollott. 2014. "Mitochondrial Reactive Oxygen Species (ROS) and ROS-Induced ROS Release." *Physiological Reviews* 94 (3): 909–50. https://doi.org/10.1152/physrev.00026.2013.