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“Characterization of GATA1 transcription factor functions in murine hematopoiesis”

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Τίτλος:

"Διερεύνηση της λειτουργίας του μεταγραφικού παράγοντα GATA1 στην αιμοποίηση του ποντικού"

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To my family……

This master’s thesis was drafted in the context of the postgraduate program “Molecular Basis of Human Diseases”.

Upon its completion I feel the need to thank some of the people I collaborated with and who played a pivotal role in its completion.

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SUMMARY

GATA1 is hematopoietic transcription factor that is known to be critical for the differentiation of several hematopoietic lineages, including erythroid cells, megakaryocytes, mast cells, eosinophils and dendritic cells. GATA1 exists as a full length protein and as a short (GATA1s) isoform that lacks the N-domain. Importantly, mutations resulting in the exclusive expression of GATA1s are implicated in human hematopoietic disorders like Diamond Blackfan Anemia (DBA) and acute megakaryoblastic leukemia (AMKL) associated with Trisomy 21 in Down Syndrome. The goal of the study is to investigate the utility of two mouse models bearing a biotin tag knocked-in the GATA1 gene locus to study the functions of GATA1 and GATA1s during the formation of the aforementioned lineages. Specifically, we tested whether the two knock-in mouse models expressed full length biotin-tagged GATA1 or biotin-tagged GATA1s in these lineages, when crossed with transgenic mice ubiquitously expressing the biotin ligase BirA which is required for biotinylation of the short biotinylatable tag fused to GATA1. In assessing biotinylation tagging of these two GATA1 isoforms in hematopoietic lineages, I optimized the purification of dendritic cells and mast cells from the bone marrow of adult mice, whereas eosinophils were isolated from the spleen of IL-5 transgenic mice. In contrast, megakaryocytes were isolated from E12.5 fetal livers. My study suggests that megakaryocytes and dendritic cells express GATA1 at such levels that it can be detected in crude protein extracts. Detection of BirA expression also suggests that bioGATA1 and bioGATA1s are likely to be *in vivo* biotinylated in these lineages. Eosinophils were also found to express detectable levels of tagged GATA1, however it was not possible to detect BirA expression or biotinylation. Mast cells, as previously described, do not appear to express GATA1 unless they are stimulated with IL-3. Thus, in this study I have successfully applied methods for the isolation of the hematopoietic cells of interest from mouse bone marrow or fetal liver cells and have identified megakaryocytes, dendritic cells and possibly eosinophils as lineages where the biotin tag knock-in GATA1 and GATA1s mouse models would be useful in studying their function.

ΠΕΡΙΛΗΨΗ

Τα ερυθροκύτταρα, μεγακαρυοκύτταρα, ηωσινόφιλα, δενδριτικά και σιτευτικά κύτταρα είναι αιμοποιητικά κύτταρα που προέρχονται από τη διαφοροποίηση προγονικών αιμοποιητικών κυττάρων. Όπως έχει γίνει γνωστό, ο μεταγραφικός παράγοντας GATA1 φαίνεται να επιτελεί σημαντική λειτουργία στο σχηματισμό τους. Πέρα από τη φυσιολογική του μορφή, ο GATA1 έχει ανιχνευτεί και σε μια μεταλλαγμένη μορφή η οποία υπολείπεται τον N-τομέα που βρίσκεται στο αμινοτελικό άκρο της φυσιολογικής πρωτεΐνης. Ο μεταλλαγμένος τύπος του GATA1 ονομάζεται GATA1s (GATA1 short) και έχει ανιχνευθεί σε σύνδρομα όπως την αναιμία Diamond Blackfan και την οξεία μεγακαρυοβλαστική λευχαιμία σε ασθενείς με σύνδρομο Down. Για τη διεκπεραίωση της παρούσας μελέτης χρησιμοποιήθηκαν τα bioGATA1 και bioGATA1s μοντέλα ποντικών όπου στο κάθε μοντέλο έχει προστεθεί ένα σήμα βιοτινυλίωσης στο αμινοτελικό άκρο της πρωτεΐνης GATA1 και GATA1s αντίστοιχα. Ο ρόλος αυτής της μελέτης είναι να εκτιμηθεί η χρησιμότητα των δυο μοντέλων ποντικών στην διερεύνηση της λειτουργίας του GATA1 κατά το σχηματισμό των προαναφερθέντων κυτταρικών τύπων του αιμοποιητικού συστήματος. Συγκεκριμένα, ποντίκια bioGATA1 και bioGATA1s διασταυρώθηκαν με διαγονιδιακό ποντίκι που εκφράζει την βακτηριακή πρωτεΐνη BirA, η οποία φυσιολογικά βιοτινυλιώνει την αλληλουχία σήμανσης από βιοτίνη που έχει προστεθεί στα διαγονιδιακά ποντίκια bioGATA1 και bioGATA1s. Εν συνεχεία, εξετάστηκε εάν οι απόγονοι εκφράζουν βιοτινυλιωμένο bioGATA1 και bioGATA1s. Αφού εξακριβώθηκε η απομόνωση και ταυτοποίηση των δενδριτικών και σιτευτικών κυττάρων από το μυελό των οστών, των ηωσινόφιλων από σπλήνα διαγονιδιακού ποντικού IL-5 που χαρακτηρίζεται από ηωσινοφιλία, και των μεγακαρυοκυττάρων από εμβρυικό συκώτι 12.5 ημερών, εξετάστηκαν τα επίπεδα GATA1, η έκφραση BirA και η κατάσταση βιοτινυλίωσης.. Τα αποτελέσματα της μελέτης οδήγησαν στο συμπέρασμα πως τα μεγακαρυοκύτταρα και τα δενδριτικά κύτταρα, εκφράζουν GATA1 ικανό να ανιχνευθεί σε πρωτεϊνικό εκχύλισμα. Επίσης, ανίχνευση BirA έκφρασης οδήγησε στο συμπέρασμα πως ο bioGATA1 και ο bioGATA1s είναι σεσημασμένοι με βιοτίνη *in vivo*. Όσο αφορά στα ηωσινόφιλα, ανιχνεύτηκε GATA1 παρόλα αυτά, δεν ήταν δυνατό να ανιχνευτεί έκφραση BirA ή βιοτινυλίωση. Τα σιτευτικά κύτταρα, φάνηκε να μην εκφράζουν ανιχνεύσιμο GATA1 παρά μόνο παρουσία κυτοκίνης IL-3. Στην παρούσα μελέτη λοιπόν, επιτυχώς απομονώθηκαν και

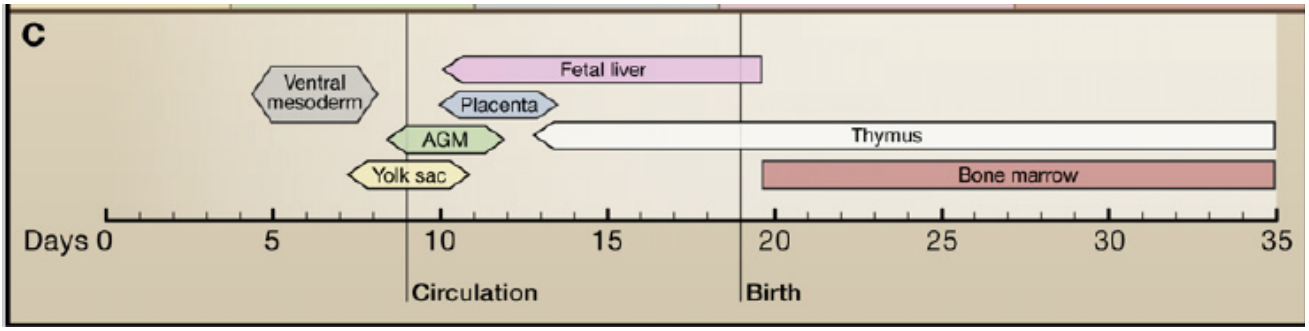
ταυτοποιήθηκαν μεγακαρυοκύτταρα, ηωσιόφιλα, δεδνριτικά και σιτευτικά κύτταρα προτείνοντας πως τα bioGATA1 και bioGATA1s μοντέλα ποντικών είναι χρήσιμα για τη μελέτη μεταγραφικού παράγοντα GATA1 στα μεγακαρυοκύτταρα, τα δενδριτικά κύτταρα και πιθανόν και στα ηωσινόφιλα.

CONTENTS

1. INTRODUCTION

1.1 Development of Hematopoiesis

Hematopoiesis is the process of generation, maintenance and differentiation of Hematopoietic Stem Cells (HSC) resulting in the formation of mature blood cells. The first wave of hematopoiesis is called primitive hematopoiesis and appears in early developmental stages of the embryo in the yolk sac (Figure 1). During this stage primitive enucleated erythroid cells are produced to support rapid embryo-growth by supplying the tissues with oxygen. The main characteristic of primitive hematopoiesis is the production of embryonic hemoglobin by primitive erythroid cells. These types of globins are absent in adults. In mammals, HSCs are generated in the ventral mesoderm. Specifically in mice, proximal mesodermal cells in the visceral yolk sac, give rise to blood cells and endothelial cells which finally form the blood islands, between gestation days 7-7.5 (Haar & Ackerman). Shortly thereafter, the endothelial cells of the blood islands synergize to generate vascular channels and then blood vessels. By gestation day 8.5 the yolk sac vessels connect with the embryo vessels, such that the blood cells and the nutrients start circulating through the embryo body. In this stage the heart starts beating. When the vessels start forming in the yolk sac blood islands, angioblasts in the embryo proper, form the major blood vessels (dorsal aortae, omphalomesenteric arter, cardinal veins, yolk sac artery and vein) (L. I. Zon, 2001). The dorsal aorta and the urogenital system form the AGM (aorta-gonad mesonephros) and become the site where HSCs are generated around day 10.5, constituting the third wave of hematopoiesis (Figure 1). In contrast with yolk sac, AGM is able to produce LT-HSC (long term HSC with sustained maintenance of high levels of HSC) (Bruijn et al., 2002). Recently, it was shown that the placenta is also a hematopoietic site, before the HSC colonization in the fetal liver at gestation day 9.



(Orkin & Zon, 2008)

Figure 1: Hematopoietic sites through gestation.

LT-HSCs generate the ST-HSCs (short term HSC) that give rise to the multineage precursors CLP (common lymphoid progenitor) and CMP (common myeloid progenitors). The CMP is the common progenitor of the MEP (megakaryocyte-erythroid progenitor) and the GMP (granulocyte-monocyte progenitor). MEPs differentiate into Red blood cells (RBCs) and megakaryocytes while GMPs differentiate into mast cells, eosinophils, neutrophils, monocytes/macrophages and dendritic cells (DCs). CLP differentiate into B-lymphoid and T-lymphoid cells, however it has also been shown that CLPs are capable to differentiate into DCs (Figure 2) (Wu, Vandenabeele, & Georgopoulos, 2001).

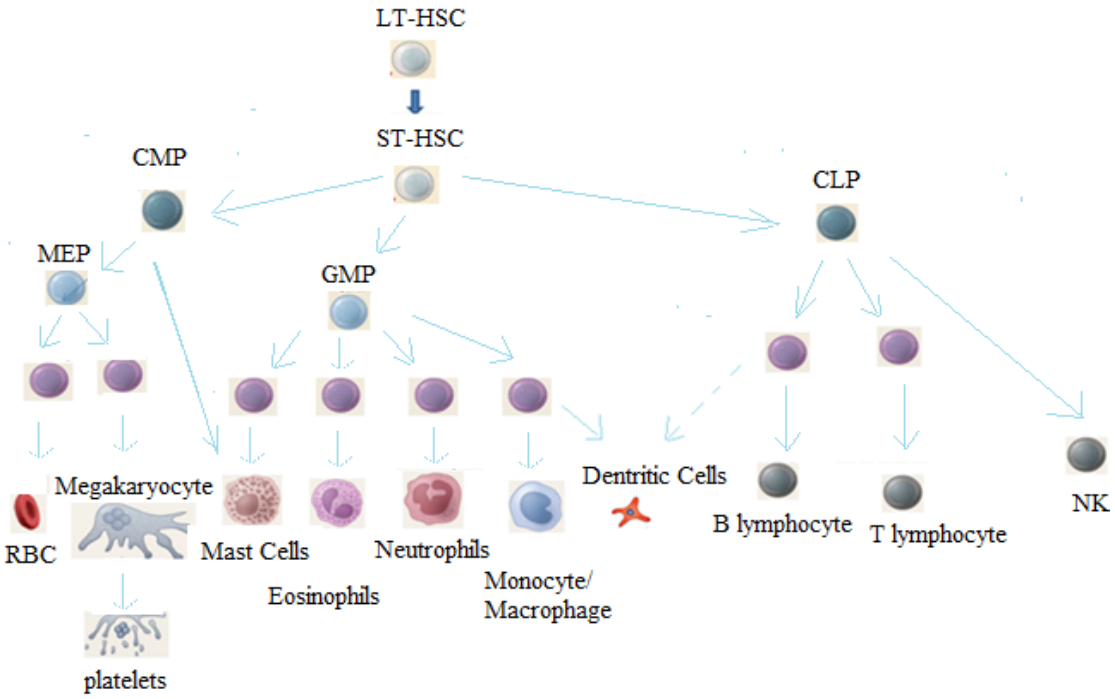


Figure 2: Generation of mature blood cells from HSC.

Megakaryocytes are large cells (diameter>80microns), with numerous granules in their cytoplasm and a large lobulated nucleus (Ackerman & Bellios, 1995). During maturation, megakaryocytes can be found as megakaryoblasts, promegakaryocytes, megakaryocytes and meta-megakaryocytes. During megakaryopoiesis, cells are enlarged as they proceed through differentiation. The meta-megakaryocyte is the last morphological stage of differentiation when the granular platelets (thrombocytes) start to be formed from meta-megakaryocytic cytoplasm and are released in the circulation (Diggs, Strum, & Bell, 1970). Each megakaryocyte is able to produce 2000-5000 platelets. Platelets are composed of rRNA and mitochondria but do not contain a nucleus. During platelet production, normal megakaryocytes undergo endoreduplication resulting in 16N ploidy, cytoplasm maturation and expansion (Garcia, 1964)(P. Vyas, Ault, Jackson, Orkin, & Shivdasani, 1999). Megakaryocytes are found in the adult bone marrow as well as in the blood islands of the yolk sac (Boe & Ph, 2011).

Eosinophils are granulocytes generated in the bone marrow and constitute only 1-3% of the blood cells (Rothenberg, Brandt, Hogan, Hogan, & Rothenberg, 2001). Their morphology is characterized by a bilobed nucleus and a granular cytoplasm (Uhm,

Kim, & Chung, 2012). Their granules contain a huge number of cationic proteins like MBP (granule major basic protein) specifically expressed in eosinophils. Eosinophils activate the immune response by acting as antigen presenting cells (APCs) and they are responsible for a variety of allergic diseases, such as asthma (B. Y. Yamaguchi et al., 2015). T-cells and B-cells are stimulated by eosinophils to promote antigen-specific immune response (H. Wang et al., 2016). In addition, eosinophils induce activation of neutrophils, mast cells and DCs (Lotfi & Lotze, 2007)(D. Yang et al., 2008)(Munitz, 2004) and maintain macrophages at adipose tissue (Locksley, Richard M.Bando et al., 2011). The IL-5 cytokine mediates eosinophilic differentiation and survival (B. Y. Y. Yamaguchi, Suda, Suda, Tominaga, & Takatsu, 1988). Thus, upon inflammatory signals eosinophils enter the circulation and reside in inflammation sites (Kim et al., 2015). CCR3 (C-chemokine receptor 3) in cooperation with the IL-5 receptor, seems to regulate eosinophil trafficking (Rosenberg, Phipps, & Foster, 2007). Transgenic mice expressing high levels of IL-5 are characterized by high levels of eosinophils in their blood and tissues (Dent, Strath, Mellor, & Sanderson, 1990).

Dendritic cells (DCs) are antigen presenting cells that are found in the circulation as immature dendritic cells and are responsible for the induction of the primary immune response. Immature DCs capture the antigen in the circulation and transfer it to lymphoid organs where the DC maturation starts with the help of activated T lymphocytes. The terminal maturation of DCs induces lymphocyte expansion and differentiation while DCs are led to apoptosis. B-cells interact with T-cells and DCs and as a result B-cells differentiate into plasma cells that produce specific antibodies. DCs are also able to fight pathogens like viruses by secretion of cytokines that recruit macrophages eosinophils and natural killer cells (Palucka et al., 2000). In mice, DCs are generated by myeloid and lymphoid precursors (Scheicher, Mehlig, & Reske, 1992)(K. Inaba et al., 1992). Myeloid and lymphoid DCs have different phenotype, localization and function (Li Wu, Chung-Leung Li, 1996)(Genetics, Corporation, & Walter, 1996)(Vremec & Shortman, 1997)(Pulendran et al., 1997). Myeloid DCs (mDCs) are usually found in the marginal zone of the spleen but migrate upon pro-inflammatory signals (Steinman, Pack, & Inaba, 1997)(Brussel, 1996). DCs can be distinguished in$B220_{pos}$ (conventional-DCs or cDCs) and $B220_{neg}$ (plasmacytoid-DCs or pDCs) due to the expression of the B220 lymphoid marker (CD45RA)(Shortman,

Liu, Walter, & Hall, 2002). cDCs are produced by bone marrow and spleen myeloid progenitors (M. Inaba, Deguchi, Hagi, & Muramatsu, 2000). cD11c$_{low}$cDCs progenitors are exported from the BM and spleen, enter the circulation and capture the antigens (Mason, Pugh, & Webb, 1981) (Cella, Engering, Pinet, Pieters, & Lanzavecchia, 1997).

Mast Cells are implicated in the first step of the immune response (Erb, Holloway, & Gros, 1996). They fight pathogens by phagocytosis, ROS and antimicrobial peptide production. Mast cells also have a key role in wound healing and in homeostasis regulation. They are capable of controlling the presence of endogenous or exogenous toxins maintaining the organism's homeostasis (Abraham & John, 2010). In addition, mast cells are associated with allergic reactions because their activation occurs after IgE binding to FcεR due to a signal cascade activated by antigens (Asai et al., 2001)(Kalesnikoff et al., 2001)(Yamasaki, Ishikawa, Kohno, & Saito, 2016). Their morphology is characterized by their small round size and cytoplasmic enrichment of granules with high concentration of histamine and heparin (Singh, Dua, Aggarwal, & Gill, 2015). Mast Cells originate from GMPs in the bone marrow (Sharada, Girish, & Umadevi, 2006). As soon as the committed precursors enter the circulation and reside in tissues, they start differentiating and become mast cells under the influence of cytokines and stem cell factor (SCF)(Okayama & Toshiaki, 2006). Mast cells can be identified as three distinct populations. The population includes mast cells that are found under the surface of the connective tissue but not attached to blood vessels. Because of the densely granulated cytoplasm, their nucleus is not visible. These cells are called "intact cells". The srcond population includes mast cells found in the upper face of connective tissue. These cells appear in irregular shape but are still granular and are called "spreading cells". The last population of mast cells are cells that secrete cytokines by losing their granules, after activation due to inflammatory response (Singh et al., 2015). However this procedure is not obligatory for cytokine secretion. Because of their degranulation, the mast cell nucleus can be seen in this stage.

1.2 Transcriptional regulation of Hematopoiesis

The generation of blood cells from HSCs requires tight regulation of lineage-restricted transcriptional programs. A number of transcription factors play a crucial

role in blood cells generation. Examples of such critical transcription factors include GATA1, FOG1, Gf1-b, RB, PU.1, EKLF transcription factors and TAL/SCL/LMO/LDB1 complex. The main focus of the thesis is the molecular basis of GATA1 functions in hematopoiesis. Below I present background information on GATA1 and related transcription factors.

1.2.1 GATA1

GATA1 also known as NFE1, Ery-1, GF-1 and NF-1 is a transcription factor encoded by the Gata1 gene located on the X-chromosome in human and mouse (Orkin, 1990). GATA1 belongs to the GATA transcription factors family which consists of six members (GATA1-6). The main characteristic of GATA factors is that they are able to bind to the DNA sequence (A/T)GATA(A/G) through two GATA-specific zinc-finger motifs (Ko & Engel, 1993)(Merika & Orkin, 1993)(Whyatt & Grosveldl, 1993). Three of the GATA family factors are implicated in hematopoiesis (GATA1, GATA2, GATA3). Both GATA1 and GATA2 play a crucial role in erythroid and megakaryocytic cells maturation. GATA1 recognizes and binds directly to regulatory regions on α- and β- globin genes (Evans, Reitman, & Felsenfeld, 1988)(Martin & Orkin, 1990)(Wall & Grosveld, 1988). GATA1 also has high affinity to regions of genes encoding heme biosynthesis enzymes (Shirihai, Gregory, Yu, Orkin, & Weiss, 2000). Heme is the molecule bound by globins that contains an iron atom that is able to capture oxygen and transport it through the circulation. GATA1-EpoR (erythropoietin receptor) promoter interaction is also established supporting that GATA1 regulates erythroid cells proliferation and survival by mediating EpoR expression (Lacombe & Mayeux, 1999)(Chiba & Kishi, 1993). Bcl-X_L anti-apoptotic protein is found to be upregulated in a GATA1-dependent manner meaning that GATA1 plays a role in differentiation of erythroid cells by preventing apoptosis through antiapoptotic protein expression (Silva et al., 2016)(B. T. Gregory et al., 1999). In addition, GATA1 knockout mice are embryonic lethal because the erythroid cells are arrested in the pro-erythroblast stage of maturation (Fujiwara, Browne, Cunniff, Goff, & Orkin, 1996). GATA2 is responsible for the self-renewal of hematopoetic stem cells and thus maintain blood lineages generation (Frelin et al., 2016) As GATA1 levels increases during erythroid cells maturation, they repress GATA2 expression allowing erythroid differentiation to proceed to completion

(Ferreira, Ohneda, Yamamoto, & Philipsen, 2005). GATA2 factor is essential for the p45 NF-E2 megakaryocyte-specific genes (Mouthon et al., 2003). GATA1 protein has three functional domains: the N-terminal Domain (N-domain), the N-terminal Zinc-finger (N-F) and the C-terminal Zinc-finger (C-F). The N-domain is a transcriptional activation domain while the zinc-fingers are related to DNA-binding. The C-F is responsible for the recognition and binding of GATA1 to the (A/T)GATA(A/G) DNA sequence while the N-F modulates the specificity of binding and stabilizes it. In addition, both zinc-fingers are implicated in GATA1 protein interactions (H. Yang & Evans, 1992)(Martin & Orkin, 1990).

GATA1 is regulated at the posttranslational level by acetylation through CBP, phosphorylation as GATA1 can be phosphorylated at seven serines, and SUMOylation as the SUMO peptide which is a ubiquitin-like peptide is bound to 137 lysine residue of GATA1, that is related to transcriptional suppression (Hung, Lau, Kim, & Weiss, 1999)(Verger, Perdomo, & Crossley, 2003). GATA1 protein levels are also regulated by protein degradation (Scholar et al., 2000). Acetylated GATA1 has been proposed to be the active form of the protein and triggers GATA1 ubiquitination targeting it to the proteasome for degradation (Hernandez-hernandez et al., 2006). As it was previously mentioned, GATA1 is able to interact with other proteins through its zinc-fingers and thus regulate different processes as a transcription factor. GATA1 can interact with, and repress the Cyclin D2 and Cdk6 genes involved in cell cycle progression and it can interact with, and activate $p18^{INK4c}$ and $p27^{Kip1}$ genes that are involved in prevention of cell cycle progress (Weiss, Yu, & Orkin, 1997). GATA1 also binds to protein complexes like P300/CBP leading to the chromatin opening (Barrett, Gustafson, Wang, Wang, & Ginder, 2004). It was also shown *in vitro* that GATA1 is able to undergo self-dimerization (Crossley & Merika, 1995).

Alternative internal translation start site on *gata1* mRNA produces a short GATA1 isoform (GATA1s) which lacks 83 amino acids in the GATA1 N-domain (Figure 3) (Rainis et al., 2003) (Calligaris, Bottardi, Cogoi, Apezteguia, & Santoro, 1995). In humans the GATA1s isoform is related to Diamond Blackfan Anemia (DBA), which is congenital anemia characterized by erythroid hypoplasia (Parrella et al., 2015). GATA1s is also associated with transient myeloproliferative disease (TMD/TL) (Maroz et al., 2013) or acute megakaryoblastic leukemia (AMKL) which occur with high frequency in Down Syndrome (DS) patients. iPSC clones from DS patients bearing a mutation resulting exclusively in GATA1s expression had a 20fold decrease

in erythroid expansion capacity (Byrska-bishop et al., 2015). Furthermore, GATA1s mutated patients suffer from erythroid hypoplasia and anemia throughout their life. It appears that GATA1s can recognize and binds to megakaryocyte-specific genes but binding to erythroid genes is negatively affected (Albuquerque et al., 2006). In addition, GATA1s has no affinity for binding to RB which is essential for erythropoiesis (Kadri, Shimizu, Ohneda, Maouche-chretien, & Gisselbrecht, 2009). In DS transient leukemia patients, GATA1s seems to promote eosinophils accumulation (Maroz et al., 2013). In mice, GATA1s mutations cause impairment of erythropoiesis while enhancing megakaryopoiesis a fact that sometimes can negatively affect embryo survival. However, most of GATA1s mutant mice are able to survive to birth and have no impairment to their hematopoiesis as adults in contrast with humans (Byrska-bishop et al., 2015).

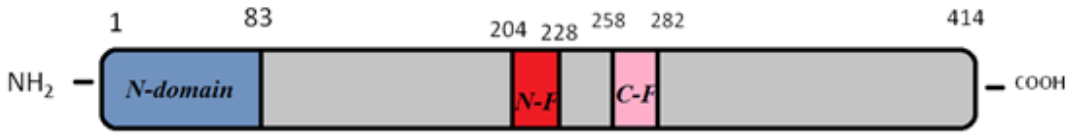


Figure 3: GATA 1 protein. GATA1short isoform is produced when the N-domain is not expressed.

1.2.2 GATA1 interacting protein partners

FOG1 (Friend of GATA1) is a multi-zinc finger GATA1 co-factor (Tsang et al., 1997). It binds to the N-terminal zinc finger of Gata1 through some of its zinc fingers (Fox et al., 1999)(Fox, Kowalski, King, Mackay, & Crossley, 1998). FOG1 null mice are lethal at gestation day E10.5-E11.5 because of severe anemia caused by defective erythropoiesis (Tsang et al., 1997). In addition, FOG1 depletion leads to loss of the megakaryocytic lineage (Tsang, Fujiwara, Hom, & Orkin, 1998).

PU.1 is a transcription factor of the ETS family. PU.1 is the master regulator of the myeloid blood lineages but it also regulates B and T lymphocyte production (Scott, Simon, Anastasi, & Singh, 1994)(Mckercher et al., 1996). PU.1 and GATA1 antagonize each other through a direct physical interaction between PU.1 and GATA1 DNA-binding (Uron, Enen, & Un, 1999)(Zhang et al., 2016). PU.1 suppression

enhances erythropoiesis, while GATA1 suppression induces myelopoiesis (Rekhtman, Radparvar, Evans, & Skoultchi, 1999)(Zhang et al., 2000).

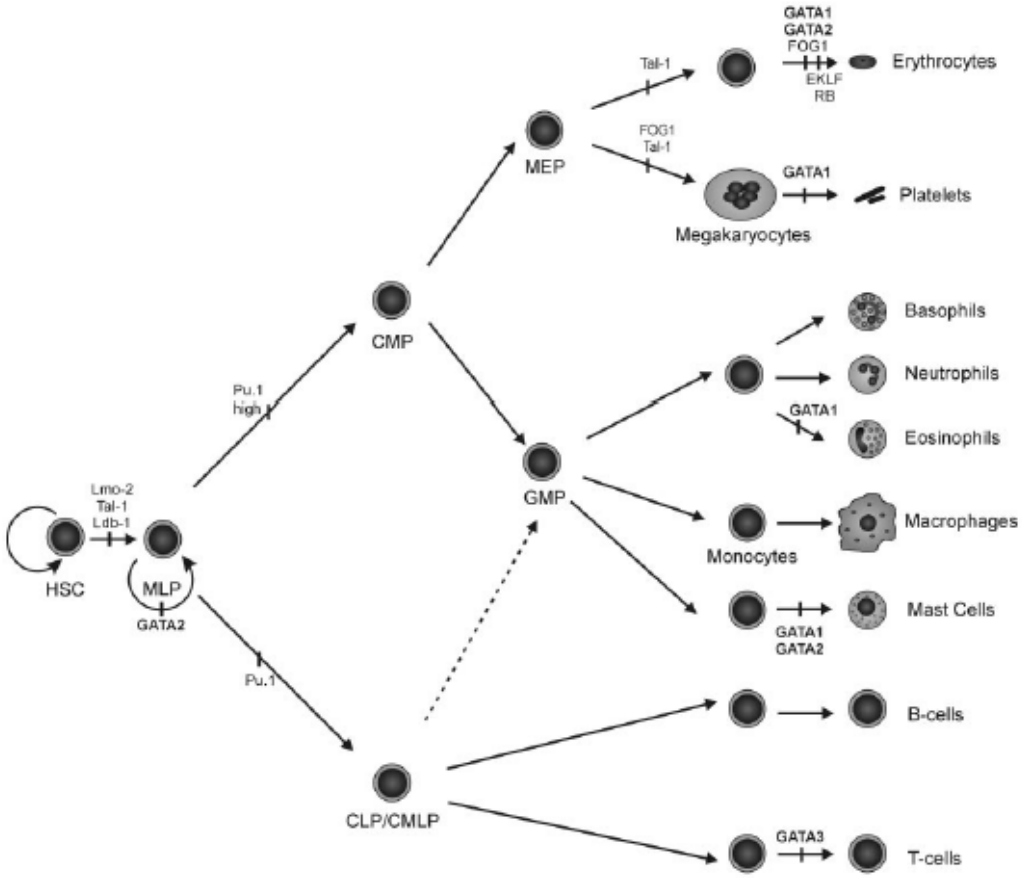
The tumor suppressor protein retinoblastoma (RB) is essential for the differentiation of blood cell progenitors. In G1 phase of the cell cycle, RB is hypophosphorylated in complex with E2F preventing the entrance in S phase. RB knockout mice are lethal because of defective erythropoiesis (Clark, Doyle, & Humbert, 2004)(Clarke et al., 1992). In addition, RB is suppressed in murine erythroleukemia cells (MEL) after GATA1 overexpression, thus preventing the transition of the cells to the S-phase (Whyatt et al., 1997).

The Kruppel-like erythroid specific factor (EKLF) is a zinc finger transcription factor, vital for β-globin expression and erythroid differentiation (Bieker, 2005). EKLF zing fingers recognize and bind to a CACC box motif found in the β-globin promoter as well as in many erythroid genes (B. R. C. Gregory et al., 1996). EKLF null mice present with no β-globin gene expression, accompanied with closed chromatin structure to the β-globin promoter region, defective expression of hemoglobin biosynthesis genes and delay in the progression of cell cycle (Wijgerde et al., 1996)(Drissen et al., 2005)(Pilon et al., 2008). It is also established that EKLF recognizes motifs in close proximity with GATA1 suggesting cooperative function between the two factors (B. R. C. Gregory et al., 1996).

Growth factor-independent 1b (Gfi-1b) is also implicated n erythropoiesis, potentially by repressing cell proliferation (Rodriguez et al., 2005). Gfi-1b also contains zing fingers and binds to mitogenic genes such as MYB in suppressing cell proliferation (Duan & Horwitz, 2003)(Doan et al., 2003). The Gfi-1b knock-out mice do not survive beyond day E15 because of defects in erythroid and megakaryocytes differentiation in the fetal liver (Seleque et al, 2002). In addition, it was shown that Gfi-1b interacts with GATA1 in repressing cell cycle genes (Rodriguez et al., 2005).

TAL1/SCL is a basic helix-loop-helix transcription factor that functions in HSCs (Porcher et al., 1996). In erythroid cells it interacts with Lmo-2/Ldb-1 and GATA1 in upregulating erythroid transcription program (Wadman et al., 1997). TAL-1/SCL knock-out mice are lethal on gestation day E9.5 because of the absence of

hematopoiesis in the yolk sac (Robb et al., 1995)(Shivdasani, Mayer, & Orkint, 1995). Thus, is suggested that TAL-1/SCL complex has a vital role in very early hematopoiesis.



(Ferreira et al., 2005)

Figure 4: Schematic representation of blood cells generation connected to transcription factors' activity.

1.2.3 GATA1/GATA1s in the process of megakaryocytes, eosinophils, mast cells and dendritic cells generation

Erythroid/ Megakaryocytes

Erythroid and megakaryocyte lineages originate from a common progenitor called MEP. Both erythroid-specific and megakaryocyte-specific genes contain GATA-binding sequences suggesting they are regulated by GATA family proteins (Evans et al., 1988)(Martin & Orkin, 1990)(Wall & Grosveld, 1988)(Shirihai et al., 2000)

(Lemarchandel, Ghysdael, Rahuel, & Romeo, 1993)(Deveaux et al., 2016)(Ravid, Doi, Beeler, Kuter, & Rosenberg, 1991)(Hickey & Roths, 1993)(Yagi, Edelhoffy, Disteches, Gerald, & Ibp, 1994). The GATA1 knockout results in early embryonic lethality due to defective erythropoiesis. In the absence of GATA1, erythroid progenitors are arrested in the pro-erythroblast stage(Fujiwara et al., 1996). Normally, GATA1 transcription factor binds to RB and EKLF resulting in activation of erythroid-specific gene expression (Whyatt et al., 1997) (B. R. C. Gregory et al., 1996). At the same time, GATA1 binds and suppresses GATA2 leading precursors to undergo erythroid differentiation. GATA1 levels of expression determine the direction of progenitor differentiation (erythroid or Megakaryocytes). During megakaryopoiesis, GATA1 is vital as GATA1 null megakaryocytes express decreased levels of megakaryocyte-specific genes accompanied with 2N ploidy of megakaryocytes instead of 16N ploidy of the wild type (P. Vyas et al., 1999). In addition, chicken and murine cell lines that overexpress GATA1 in megakaryocyte progenitors, were characterized by suppression of myeloid markers and increased differentiation rate (Kulessa, Frampton, & Graf, 1995). Interaction between GATA1 and CBfβ/RUNX1 complex was found to activate megakaryocyte lineage development (Elagib et al., 2003). Moreover, deficiency of GATA1 in megakaryocytes was found to have similar results with the deficiency of FOG1 and NF-E2 (B. P. Vyas, Ault, Jackson, Orkin, & Shivdasani, 2016). NF-E2 is essential for the terminal differentiation and platelet production of megakaryocytes (Shivdasani, Rosenblatt, et al., 1995),while FOG1 induces the megakaryocyte differentiation of 416B myeloid cell line(Tsang et al., 1997). GATA1 also interacts with FOG1, an essential interaction for megakaryocytes development as the complex interacts with other transcription factors, such as Fli-1 which is vital for the megakaryocytic development (X. Wang et al., 2002). Hence, it is established that GATA1 transcription factor plays an important role in both erythropoiesis and megakaryopoiesis.

Eosinophils

The first evidence of acorrelation between GATA1 and eosinophils was reported after the observation that enforced intermediate expression of GATA1 in myb-ETS-transformed chicken myeloblasts induced their reprogramming into eosinophils (Kulessa et al., 1995). Similar results were obtained when GATA1 was force-

expressed in human primary myeloid progenitors, turning them into eosinophils (Hirasawa et al., 2002). GATA1 binding sites are located in eosinophil-specific genes. For example, a double GATA binding site is found in intron 1 of the CCR3 gene (Rothenberg & Hogan, 2006)(Zimmermann, Colyer, Koch, & Rothenberg, 2005) and in the MBP promoter (B. Y. Yamaguchi et al., 2015), suggesting that GATA1 is involved in eosinophilic gene regulation. A double GATA1 site is also located downstream of the GATA1 gene (Du et al., 2002) which, when deleted caused depletion of the eosinophilic lineage (Yu et al., 2002). Normally, FOG1 a GATA1 partner in erythroid cells and megakaryocytes, is downregulated by C/EBP in order for eosinophilic differentiation to proceed (Yu et al., 2002). However, C/EBPb expression alone does not induce eosinophil differentiation (Muller, Kowenz-leutz, Grieser-ade, Graf, & Leutz, 1995). When FOG1 is forcibly expressed in avian eosinophils where it is normally absent, eosinophils are reprogrammed to multipotent cells (Querfurth, Schuster, Kulessa, Crispino, & Doderlein, 2000). Hence, the disruption of GATA1-FOG1 interaction is essential for GATA1 functions in eosinophil differentiation. Other studies demonstrate that PU.1 and GATA1 are cooperating in eosinophilic lineage regulation (Du et al., 2002). In addition, studies in DS patients suffering from transient leukemia, showed that GATA1s mutations are responsible for the extensive eosinophils numbers present in their blood. This is because GATA1s is incapable of suppressing MYC and E2F target genes which promote proliferation (Maroz et al., 2013). Thus, the N-domain of GATA1 protein which is absent in GATA1s seems to play an essential role in eosinophilic lineage differentiation.

Dendritic cells

Dendritic cells mainly originate from the GMP, a progenitor that can give rise to mast cells, eosinophils, neutrophils and macrophages. GATA1 has a distinct role at the GMP stage where it seems to determine the cell fate between mast cells and dendritic cells. Studies in bone-marrow derived DCs suggested that the cell fate between MCs and DCs is determined by PU.1 expression. The proposed mechanism is that GATA1 antagonizes the PU.1 interaction with the IRF4/8 promoters thus inhibiting the PU.1 recruitment and suppressing dendritic cells formation. Another way of PU.1 suppression by GATA1 could be that GATA1 affects PU.1 protein levels in a translation, post-translation, or degradation-specific manner preventing PU.1 binding

to IRF promoters (Shimokawa et al., 2010). In addition to the above information, GATA1 is also vital for the cell fate between macrophages and dendritic cells, by regulating again PU.1 expression in latter cells (Gutierrez et al., 2007). In conclusion, appears to also have a role in DC differentiation, which is not yet well-understood.

Moreover, GATA1 is also implicated in DC activation after LPS stimulation in a more distinct way. GATA1 is expressed in all DC precursors and in all differentiated DC subtypes and is vital for their survival (Gutie et al., 2016). GATA1 in DCs can also be detected in the cytoplasm and the nucleus of mature mDCs and pDCs where it is expressed in a steady-state manner (Kozma et al., 2010). During DCs activation, GATA1 expression is upregulated and so is the Ifng (interferon γ) gene expression. Interestingly, the Ifng promoter has been found to contain GATA1 binding sites (Gutierrez et al., 2007).

Taken together, it is clear that GATA1 is implicated in both differentiation and activation of dendritic cells.

Mast cells

It has been reported that GATA binding sites are located in the regulatory regions of genes specifically expressed in mast cells, such as carboxypeptidase A, chymase, histidine carboxylase, α and β FcεR subunits, IL-4 and IL-13 (Nishiyama, Yokota, Okumura, & Alerts, 2016)(L. Zon et al., 1991)(Yatsunamilg et al., 1994)(Caugheys, Zerweck, Vandersliceq, & Francisco, 1991)(Kwan, Powell, Nachman, & Brown, 2005). Mast cell precursors in BM express GATA1/2, SCL/Tal and PU.1 transcription factors (Okayama & Toshiaki, 2006). GATA2 is vital for mast cell development, as GATA2-deficient differentiated ES cells are unable to give rise to mast cells (Tsai, Orkin, Hughes, Farber, & Medical, 2016).

Mast cell activation, involves degranulation through which mast cells lose their granules in order to secrete cytokines to support immune response. Degranulation of MCs seems to depend on GATA's targeting the PCL-γ1 gene which plays a key role in the differentiation stage of most hematopoietic cell lineages (Ishijima, Ohmori, Uenishi, & Ohneda, 2012a). However, GATA1 is undetectable in non-activated mast cells. Notably, BM mast cells express GATA1 after addition of stem cell Factor and IL-3, which are cytokines that provoke mast cell activation (Harigae et al., 1998).

It has been suggested that GATA1 is essential only for MCs degranulation process. This fact was supported by experiments that showed that GATA1 repression could affect only MCs activation but not their formation (Yamamoto et al., 2014).

2. AIM OF THE STUDY

2.1 Background

Research in the lab of Dr. Strouboulis is primarily focused on the elucidation of the molecular basis of GATA1 functions in hematopoiesis and how these may be altered in the case of the GATAshort isoform which has been implicated in hematological disease. To this ends, the Strouboulis lab has generated two mouse models whereby a biotinylatable tag has been knocked-in in the first start codon in exon 2 of the mouse GATA1 gene locus, or in the second start codon in exon 3 (Figure 5).

Figure 5: Biotin tag knock-in in GATA1 gene locus. Biotin tag knock-in in exon II where the first start codon exists, or in exon III where the second start codon exists.

In this way, physiologically expressed full-lengthGATA1 protein or GATA1s protein lacking the N-terminal-most 83aa are tagged. Crossing the GATA1 and GATA1s knock-in mice with a transgenic mouse that has been generated in the Strouboulis lab which ubiquitously expresses the E.coli BirA biotin ligase, should result in the specific *in vivo* biotinylation of the tagged proteins. Biotinylation tagging of GATA1 or GATA1s can then be used for the high affinity isolation of protein complexes or in place of antibodies in ChIP assays by binding nuclear extracts or formaldehyde crosslinked chromatin, respectively, to streptavidin beads (Rodriguez et al., 2006) (Kolodziej et al.,2009) (Figure 6). Indeed, it was shown in mouse fetal liver derived erythroid cells from knock-in mice crossed with BirA transgenic mice that tagged GATA1 and GATA1s were efficiently biotinylated (Figure 7), thus suggesting that the overall strategy of employing biotinylation tagging to study GATA1 functions in hematopoiesis is feasible.

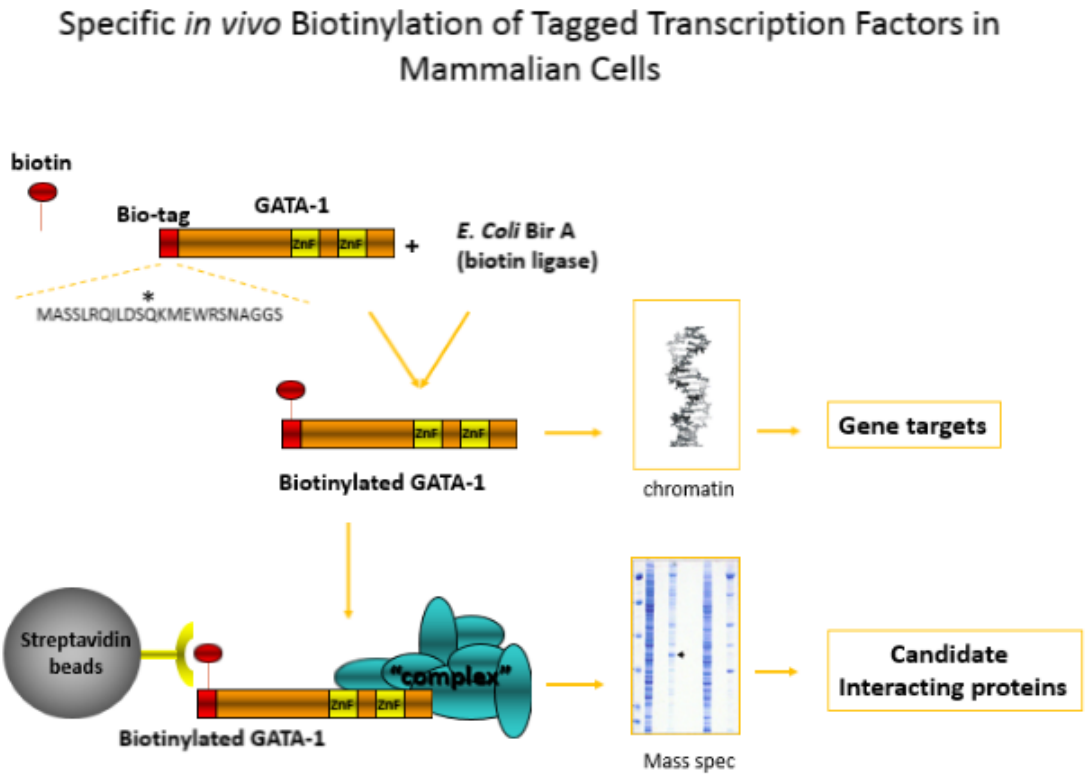


Figure 6: *In vivo* bio-tagged GATA1 or GATA1s is biotinylated when co-expressed with BirA biotin ligase. Then, using streptavidin, GATA1 or GATA1s complexes characterization can be achieved either by chromatin immunoprecipitation or by Mass Spectrometry analysis.

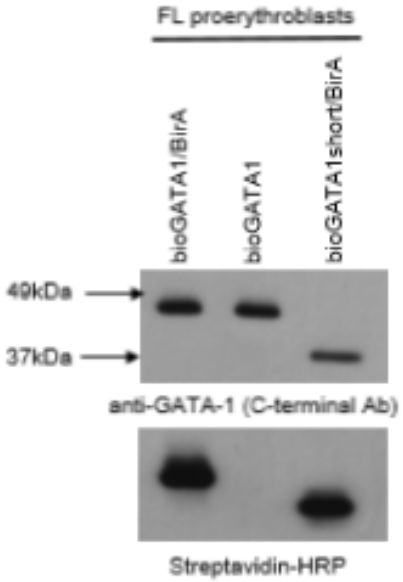


Figure 7: Western blot of fetal liver proerythroblasts. In the first blot GATA1 and GATA1s isoforms are detected with anti-GATA1 for the C-terminal domain. Streptavidin-HRP detects only bio tagged GATA1 and bio-tagged GATA1s that are co-expressed with BirA biotin ligase compared with control where BirA is absent, suggesting that bio-tagged GATA1 in fetal liver proerythroblasts is efficiently biotinylated.

2.2 Specific aim of the study

The expression of tagged GATA1 or GATA1s, of BirA and consequently, biotinylation efficiencies in hematopoietic lineages where GATA1 is normally expressed, other than erythroid cells, have not been investigated. Thus, the **specific aim** of this study is to assess the utility of the biotin tag GATA1 and GATA1s knock-in mouse models in studying GATA1 functions across different hematopoietic lineages, namely, in megakaryocytes, eosinophils, mast cells and dendritic cells. To this end, I first attempt to establish reproducible and robust methods for isolating and expanding *in vitro* sufficient numbers of hematopoietic cells from the aforementioned hematopoietic lineages, followed by the assessment of tagged GATA1 or GATA1s expression, of BirA expression and of GATA1 biotinylation efficiencies in these lineages.

3. MATERIALS AND METHODS

3.1 Chemicals and Cells culture Materials

Chemicals:

EDTA, Tris, HCl, NaCl, Phenol /Chloroform, PBS, Ammonium Chloride, HEPES, Glycerin, Glycine, Tween20, SDS, Nonidet P40 were purchased from AppliChem.

Cells culture Materials:

RPMI, IMDM, FBS, Non essential amino acids, 2-mercaptoethanol, L-glutamine, Sodium pyruvate, Penicillin/Streptomycin (P/S), Hepes were purchased from Gibco.

3.2 BirA mouse

A transgenic mouse that ubiquitously expresses the 3xHA tagged BirA biotin ligase (Papadopoulos and Strouboulis unpublished) under the promoter of hnRNPA2 housekeeping gene (Katsantoni et al., 2007) was previously generated in the Strouboulis lab.

3.3 bioGATA1 mouse

A biotin tag GATA1 knock-in mouse model has been generated in the Strouboulis lab by knocking in a short biotinylatable tag (bio-tag) in the first start-codon of the GATA1 gene locus, such that all endogenously expressed full length GATA1 protein is tagged (Karkoulia and Strouboulis unpublished). When crossed with the BirA mouse, efficient biotinylation of the endogenously expressed GATA1 protein is observed in fetal liver derived hematopoietic cells.

3.4 bioGATA1s mouse

A second knock-in mouse model was generated in which the GATA1s isoform was tagged with the bio-tag, such that all endogenously expressed GATA1 protein corresponds to tagged GATA1s (Karkoulia and Strouboulis unpublished). When crossed with the BirA mouse, efficient biotinyltion of the endogenously expressed GATA1s protein is observed in fetal liver derived hematopoietic cells.

3.5 IL-5 mouse

This transgenic mouse model was generated by expressing the mouse IL-5 gene under the control of the human DC2 Locus Control Region (Lang et al., 1988) resulting in eosinophilia (Campbell et al., 1988)(Dent et al., 1990)(Cammpell et al., 1988). IL-5

mice presenting with eosinophilis and enlarged spleens were crossed with BirA/biotinGATA1 and BirA/biotinGATA1short in order to facilitate isolation of eosinophils from the spleen.

3.6 Genotyping/DNA extraction

DNA was extracted from the tails of newborn mice, using Tail lysis buffer (100mM Tris/HCl pH=8.5/5mM EDTA/0.2% SDS/200mM NaCl) and incubated with Proteinase K (5μl in 0.5ml of 10mg/ml stock) overnight at 56^{o}C. Samples were centrifuged at 13000rpm, at room temperature (RT) for 20min. The supernatant was collected and extracted with 0.5ml Phenol/Chloroform with the addition of 10% 3M NaAc to 10% v/v, mixed throroughly and centrifuged at 13000rpm, at RT for 5min. The aqueous phase was transferred in a new eppendorf containing 0.5ml isopropanol and left at -20^{o}C for more than 30min. The DNA was pelleted by centrifugation at 13000rpm, at 4^{o}C for 15min. The pellet was washed with 70% ETOH, centrifuged again as above and left to air dry. The DNA pellet was dissolved in 100μl ddH2O.

3.7 PCR

- **bioGATA1 PCR**

PCR for screening fort the biotin tag sequence in GATA1 knock-in mice was performed with Taq Polymerase in 1xTaq buffer, 0.12mM dNTPs, $MgCl_2$ 1.5mM, 250nmoles Forward (ATCAAAGCTTACCACCATGGCCatggccacctatgacgtc) and 250nmoles Reverse (TCAGGTCGACTCATTATTTTTCTGCACTACGC) primers (Minotech). PCR reaction was carried out using the following conditions : 95^{o}C 4min, 35 cycles of 95^{o}C 1min /58^{o}C 1min/ 72^{o}C 1min 30s and one cycle of 72^{o}C for 7 min.

- **bioGATA1short PCR**

PCR for screening for the biotin tag sequence in GATA1short knock-in mice was performed with Taq Polymerase in 1xTaq buffer, 0.12mM dNTPs, $MgCl_2$ 1.5mM, 250nmoles Forward (TCACAGGTTCAACCCCAGTG) and 250nmoles Reverse (GTTGAGGCAGGGTAGAGTGC) primers (Minotech). PCR reaction was carried out using the following conditions: 94^{o}C 3min, 30 cycles of 94^{o}C 30s /58^{o}C 45s/ 72^{o}C 30s and one cycle of 72^{o}C for 2 min.

- **BirA PCR**

PCR for screening of BirAsequence was performed with Taq Polymerase in 1xTaq buffer, 0.12mM dNTPs, $MgCl_2$ 1.5mM, 250nmoles Forward (GAGAGAGCAGCCACTGCCCAAATAGGTG) and 250nmoles Reverse (TCTTTGTGTCTTTCTCAGCTTGCCTTCTG) primers (Minotech). PCR reaction was carried out using the following conditions: 95^{o}C 3min, 35 cycles of 95^{o}C 30s /57^{o}C 30s/ 72^{o}C 30s and one cycle of 72^{o}C for 7 min.

- **Il-5 PCR**

PCR for screening of IL-5 transgenic mice sequence was performed with Taq Polymerase in 1xTaq buffer, 0.12mM dNTPs, $MgCl_2$ 1.5mM, 250nmoles Forward (ACCTCATCGCCTCCCAAAAT) and 250nmoles Reverse (GGTGATCTACCTGCTTTGGCC) primers (Minotech). PCR reaction was carried out using the following conditions: 95^{o}C 3min, 30 cycles of 95^{o}C 45s /56^{o}C 40s/ 72^{o}C 30s and one cycle of 72^{o}C for 7 min.

PCR products were separated by electrophoresis in 2% agarose gel and their visualization was enabled by Ethidium Bromide staining.

3.8 Dendritic Cells isolation-culture-harvesting

Femurs and tibia were extracted from a dissected mouse. Attached muscles and bones were cleaned by placing them first in FACS medium then in 70% ETOH and finally in PBS. The bones were crashed in a mortar so the bone marrow was released. Cells were cultured at 37^{oC} under 5%CO_2 on T75 flasks, using RPMI (GIBCO), 1%P/S,FBS, 2-mercaptoethanol 5x10^{-5}M (GIBCO). According to the GMCSF protocol cells were cultured using 20ng/ml of GMCSF (Peprotec 213-13) (0.5x10^{6}cells/ml). To induce DC activation cells were stimulated with LPS on Day 8 and were harvested after 24hrs. According to the Flt3 protocol, bone marrow cells were incubated in 20ml hypotonic buffer (16mM Tris pH=7,6 /100mM Ammonium Chloride) and then cultured in the presence of 250ng/ml Flt3 (Peprotec 250-31) (10^{6}cells/ml). To induce DCs activation cells were stimulated with LPS on Day 8 and were harvested after 24hrs.Cells were seeded on slides and the rest were used for protein extraction.

3.9 Mast Cells isolation-culture-harvesting

Femurs and tibia were extracted from a dissected mouse as above. The single cell suspension was cultured at 37^{o}C under 5%CO_2 on T75 flasks, using in 50mlRPMI (GIBCO)/10%FBS/1% L-glutamine/1%P/S/25mM Hepes/1% Sodium Pyruvate (GIBCO)/1% Non essential amino acids (GIBCO)/2-mercaptoethanol 5x10^{-5}M (GIBCO).Mast cells differentiation was induced using 30ng/ml IL-3 (Peprotec 213-13). The culture was maintained at 10^{6}c/ml. Medium was changed twice a week. On week 4 the cells were harvested by making 2-3 washes with PBS at 1500 rpm, RT. Cells were seeded on slides and the rest were used for protein extraction.

3.10 Eosinophils isolation

An enlarged spleen from a transgenic IL-5 mouse was dissected and single cell suspension was made in FACS medium. The cells were centrifuged at 1000 rpm 5min 4^{o}C and resuspended in FACS medium to be counted. Cells were resuspended in 2x10^{8} cells/ml with 10μg of Gr1/Ly6c antibody (BD Pharmagen 553123), B220 antibody(BD Pharmagen 553084), Ter119antibody (BD Pharmagen 55367), CD2 antibody (BD Pharmagen 553109) and rotated at 4^{o}C for 45min. After 2 washes with PBS/0.5%BSA/2mM EDTA the cells were resuspended in 2x10^{8} cells/ml in the same buffer. Then 50μl of goat anti-rat IgG Miltenyi beads (Miltenyi 130048501) per mouse were added in the sample and rotated for 30min at 4^{o}C. 2x10^{8} cells were resuspended in 3ml PBS/0.5%BSA/2mM EDTA and were passed through LD Columns (Miltenyi 130-042-901) that are designed for stringent depletion of cells. The clear flowthrough contains eosinophils. 5x10^{4}cells were seeded in slides and the rest were used for protein extraction.

3.11 Megakaryocytes isolation-culture-harvesting

Fetal livers from E12.5 mouse embryos were dissected and homogenized in 2ml of IMDM (GIBCO) 10%FBS 1 % P/S. Single cells were cultured in 3ml IMDM (GIBCO)-10%FBS-1 % P/S-50ng/ml TPO (Peprotec 315-14) per fetal liver. On day 5 megakaryocytes were harvested by making three washes with PBS, 7000rpm. Mature megakaryocytes were harvested from the 3% BSA phase of the BSA gradient and washed once as above. 5x10^{4}cells were seeded on slides and the rest were used for protein extraction

3.12 May Grunwald/Giemsa staining

Slides were stained for 3min in May Grunwald and then washed in PBS. After the slides were dried, they were stained for 15min in Giemsa (diluted 1:20 in H_2O) and then washed in H_2O.

3.13 High Salt Protein extraction

Cells were diluted in HEPES/KOH pH=7.9 10mM/$MgCl_2$ 1.5mM/KCl 10mM/DTT 0.5mM (500µl in 4.3 x 10^7 cells), incubated on ice for 10min and then vortexed. The cytoplasmic fraction is isolated as the supernatant following centrifugation at 13000rpm, 30s, 4°C. The pellet is resuspended in HEPES/KOH pH=7.9/20mM/$MgCl_2$ 1.5mM/NaCl 420mM/EDTA 0.2mM/Glycerin 25%/DTT 0.5mM (120µl in 4.3 x 10^7 cells) and left on ice for 20min. The nuclear extract was isolated as the supernatant after centrifugation at 13000rpm, 15min, 4°C.

3.14 Bradford

2µl of protein extract was diluted in 798µl ddH_2O and 200µl Bradford (Bio-Rad). The absorbance is measured at 595nm. Based on a BSA curve, sample concentration is measured by the ratio $\frac{SampleODat\ 595nm}{0.0338}$.

3.15 Immunoprecipitation

Day 1: 150µl of Protein Sepharose beads –in two separate tubes, 100µl for the IgG control sample and 50µl for the IP sample- per 500µg of protein were equilibrated by washing them 3x with 1x PBS at room temperature. Both tubes were washed 2x with 1ml 100mM sodium citrate pH 5.0. 10µg of antibody and 10µg of IgG, each diluted in 1ml HENG buffer were added to the beads of the IP tube and the IgG tube, respectively. Tubes were incubated for 2hrs at room temperature in a rotating wheel. Beads were washed 2x with 1ml 100mM sodium citrate pH 5.0, followed by a wash with 1ml 200mM Triethanolamine Hydrochloride pH 8.2. 1ml of freshly made ice cold DMP Buffer was added and samples were incubated for 30min at room temperature with rotation. Beads were washed with 1ml 50mM Tris pH 7.5 and incubated in 1ml 50mM Tris pH 7.5 for 15min at room temperature with rotation. Beads were washed 3x with PBS-0.001% Tween20. At this point different procedures were followed for the two tubes: the IP beads were incubated on ice in the last wash buffer (PBS-0.001% Tween20) for 1hr while the IgG beads were blocked with 1ml

HENG and 200 µg/ml chicken egg albumin for 1hr at room temperature with rotation. The IP beads were then blocked with 1ml HENG and 200 µg/ml chicken egg albumin for 1hr at room temperature with rotation and in parallel the protein extracts were diluted 1:3 in ice cold HENG Buffer to adjust the salt concentration to 150mM KCl (essential for efficient binding) and were added to the blocked IgG beads. The IgG tube was incubated for 1hr at 4°C in a rotating wheel. The IP tube supernatant was discarded and the IgG supernatant, i.e the extracts, was transferred to the IP tube for overnight incubation at 4°C with rotation. The IgG beads were washed 5x 5min with HENG Wash buffer at 4°C with rotation, rinsed 2x with PBS and the protein bound to the beads was eluted by resuspension in 1x Laemmli buffer and incubation for 10min at 80°C. Tube was stored at -20°C as the IgG control sample.

Day 2: The IP tube supernatant was transferred to a separate tube in order to be used as the supernatant sample. Laemli buffer was added and the tube was incubated for 10min at 80°C. The IP beads were washed 5x 5min with HENG Wash buffer at 4°C with rotation, rinsed 2x with PBS and the protein bound to the beads was eluted by resuspension in 1x Laemmli buffer and incubation for 10min at 80°C.

HENG Buffer: 10mM HEPES-KOH pH 9, 1.5mM $MgCl_2$, 0.25mM EDTA, 20% Glycerol and 1% protease inhibitors

HENG Wash Buffer: 250mM KCl and 0.3% NP40 in HENG Buffer and 1% protease inhibitors

Protease inhibitors were added fresh prior to using the buffers.

DMP Buffer: 0.01037g DMP powder in 2ml of Triethanolamine Hydrochloride pH 8.2

3.16 TCA

100% cold TCA was added to protein samples to a final concentration of 20%. The sample was then incubated on ice for 30min and centrifuged at 13000rpm, 4^oC. The pellet was washed once with ice cold 1% TCA and then once more with ice cold

acetone. The protein pellet was left to dry and then diluted in 1x Laemmli and boiled at 80^{o}C for 10min and loaded on a gel for SDS-PAGE.

3.17 Immunoblot Analysis

50μg of protein extracts per sample were loaded into the wells of SDS-PAGE gels along with molecular weight marker. The proteins were then transferred to Nitrocellulose Membranes 0.45μm (GE Healthcare 10600002). The efficiency of the transfer was examined using Ponseau S staining. The membrane was then blocked for an 1hr in 5%BSA/PBS-0.01%Tween20 or 5% fish skin gelatin /PBST and then was incubated overnight at 4^{o}C with the 1st antibody (GATA1 N6 rat antibody -Santa Cruz sc-265 / GATA1 M20 goat antibody1:2000 in 2% BSA/PBST -Santa Cruz sc-1234 / HA rabbit antibody 1:2000 in 2% BSA/PBST -Santa Cruz sc-805 / EKLF rabbit antibody 1:2000 in 2% BSA/PBST) or Streptavidin HRP 1:10000 in 2% gelatin-fish skin/PBST (Perking Elmer NEL750001EA). The next day incubation with secondary HRP (Horseradish peroxidase) antibody takes place for an 1hr (anti-rat HRP -Santa Cruz sc-2006 / anti-rabbit HRP -Santa Cruz sc-2004 / anti-goat HRP -Santa Cruz sc-2020) at RT. For signal development, a chemilluminescence substrate (ECL 1:1) (Healthcare W9695573) was used and the image was acquired using darkroom development techniques.

3.18 WB membrane Stripping

Membrane incubation at 60^{o}C for 30min, with Stripping buffer(SDS 20% 5ml/Tris pH=6,8 3.125ml/2-Mercaptoethanol (gibco) 352μl/ ddH_2O 41.523ml). Multiple PBS washes take place before blocking and incubation of the membrane with the antibody.

4. RESULTS

4.1 Megakaryocytes

Megakaryocytes verification

Megakaryocytes are generated in the fetal liver of mice. In order to assess whether tagged GATA1 was biotinylated in megakaryocytes *in vivo*, megakaryocytes were generated from E12.5 fetal livers cultured in IMDM/10%FBS/1%P/S and 50ng/ml thrombopoietin (TPO) (Villeval et al, 1997). Thrombopoietin (TPO) is a cytokine that induces the proliferation and maturation of megakaryocyte progenitors through its binding to the c-Mpl receptor, thereby promoting a signaling cascade increasing the expression of megakaryocyte-specific genes. After 5 days of culture, differentiated megakaryocytes were isolated by BSA gradient centrifugation as large lobulated cells and were stained with May-Grunwald/ Giemsa (Figure 8A). Microscopic observation before centrifugation revealed the presence of both megakaryocytes and erythroid cells. However, microscopic observation after BSA gradient showed that erythroid cells were removed and in this way we verified megakaryocytes' isolation. Specifically on the right side of Figure 8A megakaryocytes can be recognized as large lobulated cells. The left picture which is captured in lower magnification, cannot clearly depict the structure of the cells.

To further support these results, Western blots using EKLF and Fli-1 antibodies were carried out. Normally, EKLF is present only in erythroid cells, whereas Fli-1 expression is megakaryocytic specific. Our results were in accordance with results obtained by microscopy. EKLF was detected only in erythroid cells as a single band, whereas Fli-1 was clearly detected as a single band only in megakaryocytes differentiated from fetal livers as outlined above (Figures 8B & 8C).

Hence, fetal liver cells cultured in presence of TPO can be used to generate megakaryocytes that can be further used for tagged GATA1 detection.

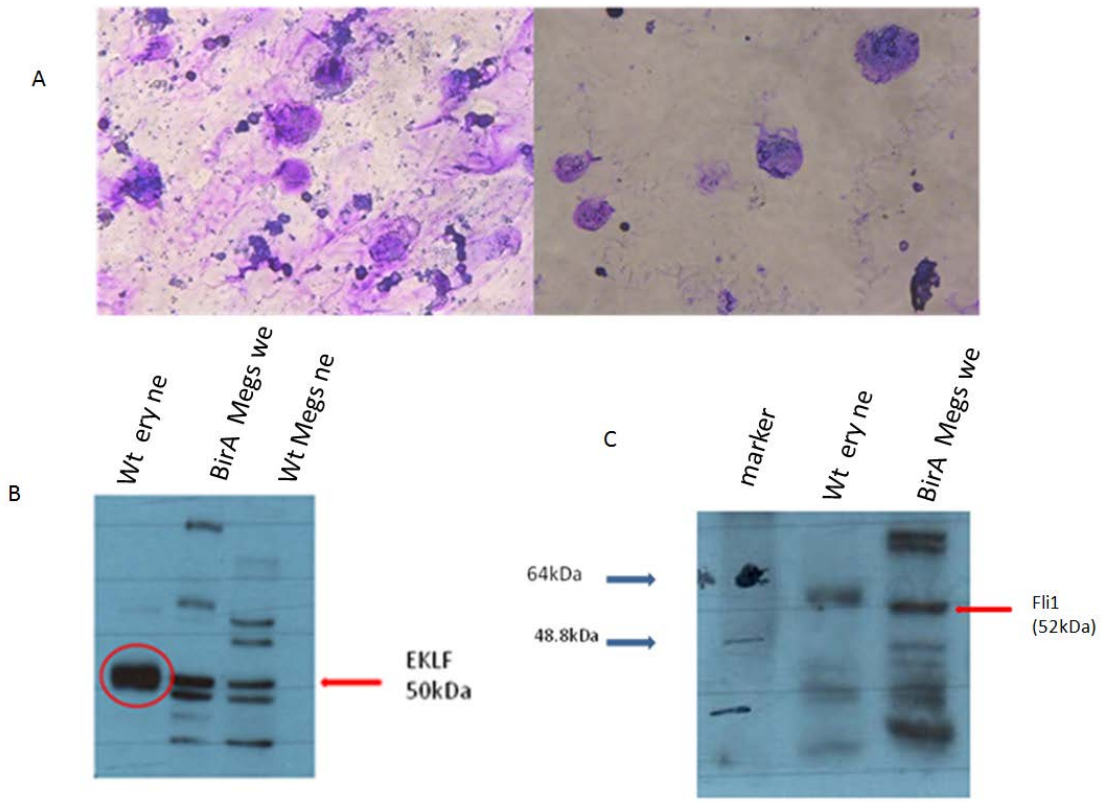


Figure 8: Megakaryocytes purification's verification. A) May-Grunwald staining. B) Western blot detection of EKLF an erythroid specific transcription factor. C) Western blot detection of Fli-1 megakaryocytic specific transcription factor.

GATA1 detection and biotinylation efficiency in Megakaryocytes

As a first effort of tagged GATA1 detection, high salt nuclear extracts of wild type megakaryocytes differentiated from fetal liver, were tested for GATA1 expression. In the light exposure in figure 9A, GATA1 can be clearly seen in nuclear extracts of megakaryocytes. Wild type nuclear extracts and whole cell extracts of megakaryocytes, show the same GATA1 band as wild type erythroid control cells. In Figure 9B, GATA1 was also detected in megakaryocytic nuclear extracts isolated from BirA transgenic mice that were used as controls. BioGATA1/ BirA extracts had undetectable GATA1 levels because of the low protein amount that was loaded. GATA1s detection is probably shown in Figure 9B, as protein band of the expected size appears only in the nuclear extracts of bioGATA1s/BirA megakaryocytes. The result is not definitive though as there was no available positive control.

BirA expression was also verified in the same samples by Western blotting in Figures 9C and 9D. However, in the bioGATA1/ BirA erythroid positive control used for the

bioGATA1s/BirA megakaryocytes blots, BirA was barely detectable. This is probably due to the low amount of erythroid nuclear extracts that was loaded in the gel to allow clear detection of GATA1, an abundant protein, and was not sufficient for BirA detection. This may have also been compounded by the stripping of the membrane to be reprobed with anti-HA for BirA detection. In contrast, in Figure 9C, BirA is detectable in the erythroid positive control as anti-HA for BirA detection was the first to be used on the membrane.

In Figure 9F, incubation of the bioGATA1s/BirA Western blot membranes with streptavidin revealed two bands in both bioGATA1/BirA nuclear extracts that were loaded. However, those results are not relaible as there was no GATA1s positive control. It is presumed though, that the second band may correspondto the GATA1s isoform as it appears to be of the expected size for GATA1s.

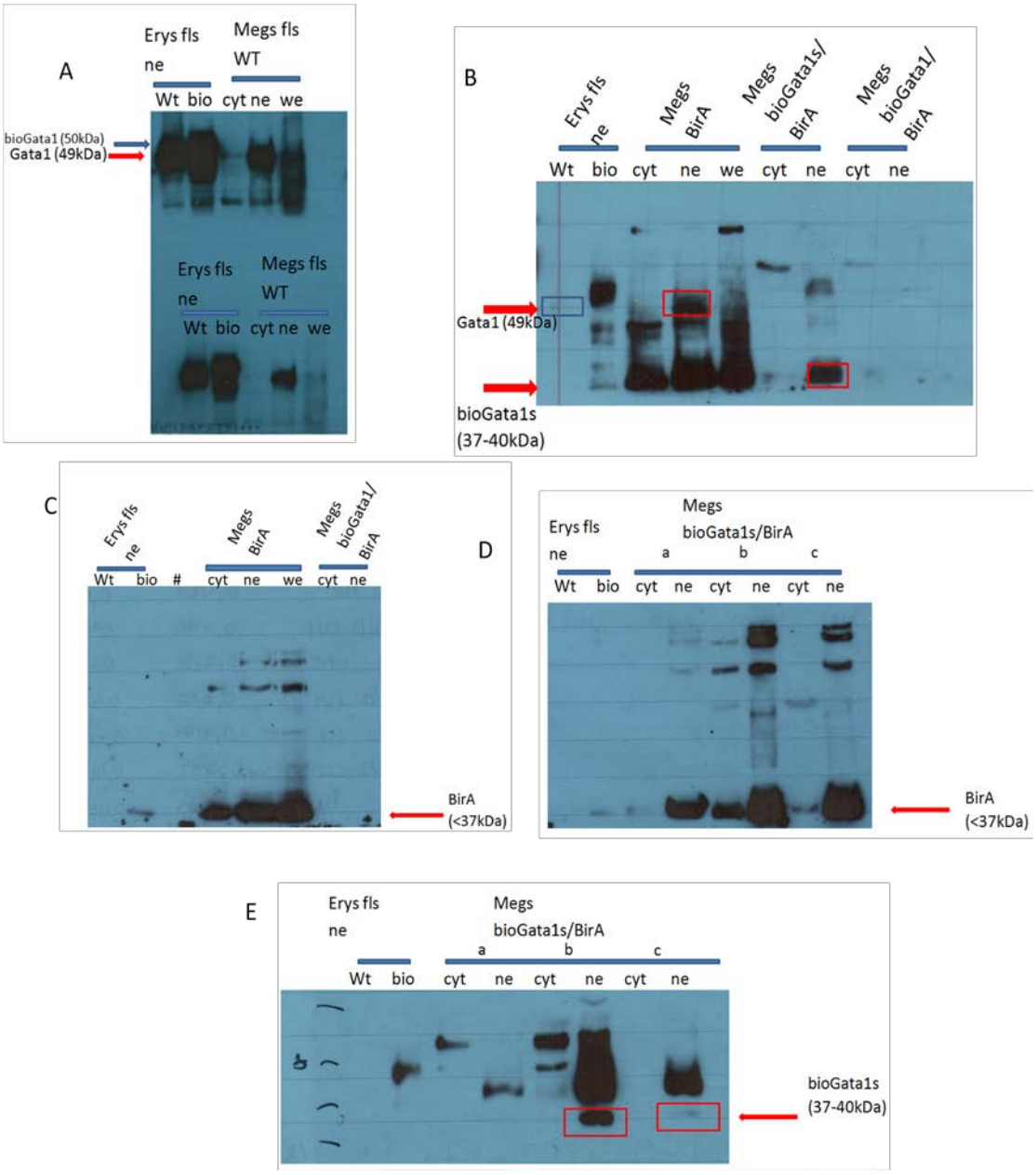


Figure 9: A) Western blot using N6 antibody for GATA1 detection in megakaryocytes of wild type mice.Erythroid cells generated from fetal livers that express abundantly GATA1 were used as controls. B)Western blot using M20 antibody for GATA1 and GATA1short detection in megakaryocytes of BirA and bioGATA1short/BirA mice respectively. Erythroid cells generated from fetal livers that express abundantly GATA1 or GATA1short were used as controls. C) Western blot for BirA detection in BirA and bioGATA1/BirA mice. Erythroid cells generated from fetal livers that express abundantly GATA1 or GATA1short were used as controls. D) Western blot for BirA detection in bioGATA1short/BirA mice. As controls erythroid cells generated from fetal livers that express abundantly GATA1 or GATA1short were used. E) Streptavidin HRP blot after stripping.

Thus, GATA1, GATA1s and BirA appear to be detected in megakaryocytes. However, I was unable to show that biotinylation is efficient. As the protocol for megakaryocyte differentiation from mouse fetal liver cells results in sufficient

amounts of protein in nuclear extracts (Table 1), the preliminary results shown here on GATA1 and GATA1s in megakaryocyte lineage, hold promise with regards to Mass Spectrometry analysis.

4.2 Eosinophils

Eosinophils verification

Eosinophils comprise 1-2% of the blood cells, thus making eosinophils isolation in sufficient quantities for tagged GATA1 detection very difficult. For this reason, GATA1 or GATA1short biotin tagged mice were crossed with IL-5 overexpressing transgenic mice, which exhibit eosinophilia. The IL-5 transgenic mouse produces high levels of the IL-5 cytokine which is responsible for eosinophilic production *in vivo* (Warren & Moore, 1988). The IL-5 transgenic mice carry more than 8 copies of the IL-5 transgene under the control of the CD2 LCR, thus causing extensive eosiniphilia. The only symptom that can be macroscopically observed in these mice is splenomegaly with a large number of eosinophils residing in spleen, bone marrow and peritoneal exudates (Dent et al., 1990). In our work, we used IL-5 transgenic mice, to isolate eosinophils from the spleen of biotin tag GATA1 knock-in/BirA mice. Eosinophils were isolated from a dissected spleen of an eosinophilic mouse using the stringent LD Column depletion, where the Column binds strictly non-eosinophils that have been labeled with non-eosinophilic antibodies. The clear flowthrough contains eosinophils. The start number of cells is 10^9 while we end up with 10^8 eosinophils per a large spleen.

The identity of the isolated cells was verified by using May-Grunwald/Giemsa staining which contains eosin, a substance that stains orange the eosinophils cytoplasm. As it was expected, eosinophils were stained orange in contrast with non-eosinophils that had no color (Figure 10).

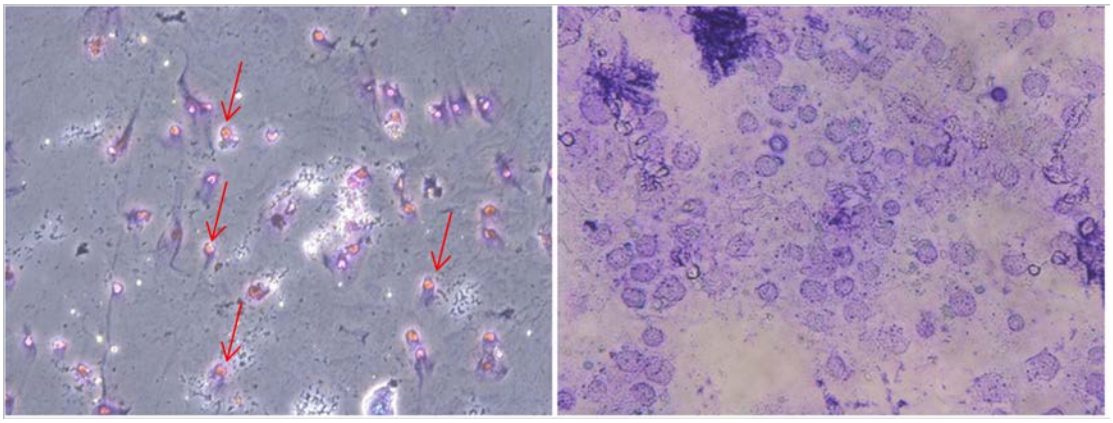


Figure 10: Eosinophils on the left side stained orange with May-Grunwald. Non-eosinophils (no color) on the right side after May-Grunwald staining.

These results show that, eosinophils can be successfully isolated from the spleens of bioGATA1/BirA/IL-5 or bioGATA1s/BirA/IL-5 transgenic mice for further work regarding biotinylation of GATA1 characterization *in vivo*.

GATA1 detection and biotinylation efficiency in eosinophils

We isolated nuclear extracts from purified eosinophils using high salt extraction (Table 1). Thus, immunoprecipitation (IP) experiments were carried out using GATA1 antibodies in order to concentrate GATA1 protein extracted from the eosinophils' nucleus. In Figure 11A, bioGATA1 is detected after IP using 500µg of whole cell extract at the expected size. In Figure 11B, a band of the expected size appears in immunoprecipitate using cytoplasmic and nuclear protein extracts isolated from bioGATA1/BirA/IL-5 transgenic mice. However, it is not clear whether the band detected of the expected size in the cytoplasmic immunoprecipitate of the bioGATA1/BirA/Il-5 mice is indeed GATA1, as the one that is detected in the cytoplasm of bioGATA1/BirA/IL-5 sample, because the IgG control was not clear. In contrast, bioGATA1 was detectable in the nuclear eosinophils immunoprecipitate from bioGATA1/BirA/IL-5.

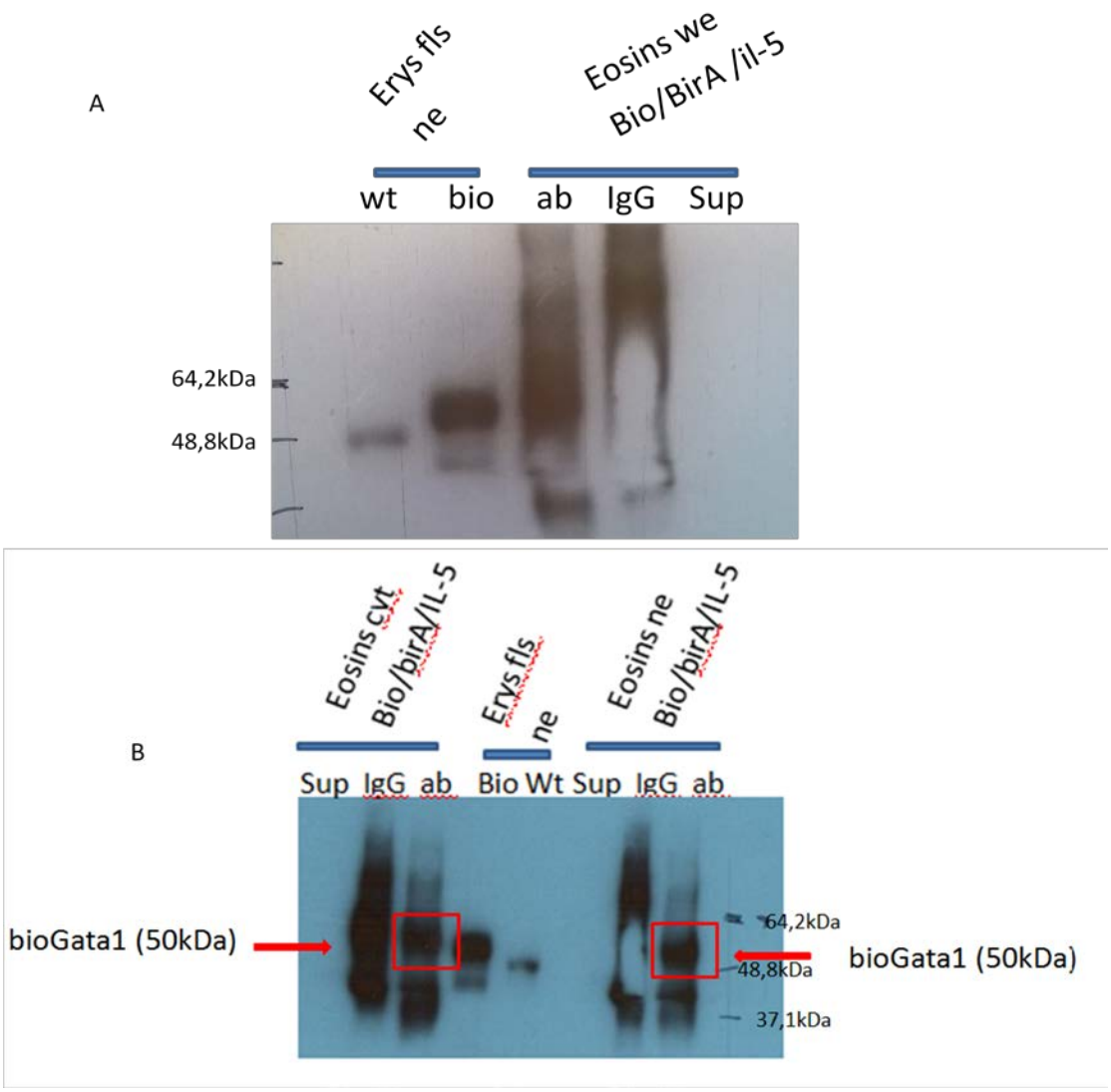


Figure 11: Protein extracts from eosinophils. Erythroid cells generated from fetal livers that express abundantly GATA1 were used as controls. A) Whole cell extracts after IP for GATA1. B) On the left side cytoplasmic protein extracts after IP for GATA1. On the right side nuclear protein extracts after IP for GATA1.

In conclusion, these results show that the spleen dissection and column depletion of spleen cells can provide us pure eosinophils, and enough protein quantity (Table 1). I estimate that we are going to need about ten enlarged spleens for the extraction of 1mg of protein making feasible to proceed to Mass Spectrometry.

4.3 Dendritic cells

Dendritic cells verification

Dendritic cells (DCs) act as antigen presenting cells (APCs), can have myeloid or lymphoid origin. GM-CSF (granulocytes/macrophage colony-stimulating factor) induces the production of myeloid DCs by myeloid-committed precursors resulting in monocyte-derived inflammatory-like DCs (K. Inaba et al., 1992)(Scheicher et al., 1992). Flt3 ligand (Flt3-L) targets myeloid and lymphoid precursors in the bone marrow (BM) increasing the production of both myeloid and lymphoid DCs (Genetics et al., 1996)(Shurin et al., 1997)(Pulendran et al., 1997).

In using the GM-CSF protocol, bone marrow progenitors were cultured for 8 days in RPMI as described by Gutierrez et al in 2007. The complete medium was RPMI, 1%P/S, FBS, 2-mercaptoethanol $5x10^{-5}$M that contained 20ng/ml of GMCSF. To induce DC activation cells can be stimulated with LPS on Day 8 and harvested after 24hr.

In using the Flt3 protocol, bone marrow cells were incubated in 20ml hypotonic buffer (16mM Tris pH=7,6 /100mM Ammonium Chloride) and then cultured in the presence of 250ng/ml Flt3 (10^6cells/ml). To induce DCs activation cells were stimulated with LPS on Day 8 and were harvested after 24hr (Gutierrez et al., 2007).

Cells were observed under the microscope and on day 8-10 it was clear from their morphology that cells that were attached to the flask were dendritic cells (Figure 12). DCs are characterized by the growth of branches projections called dendrites. Thus, they can be used for GATA1 detection.

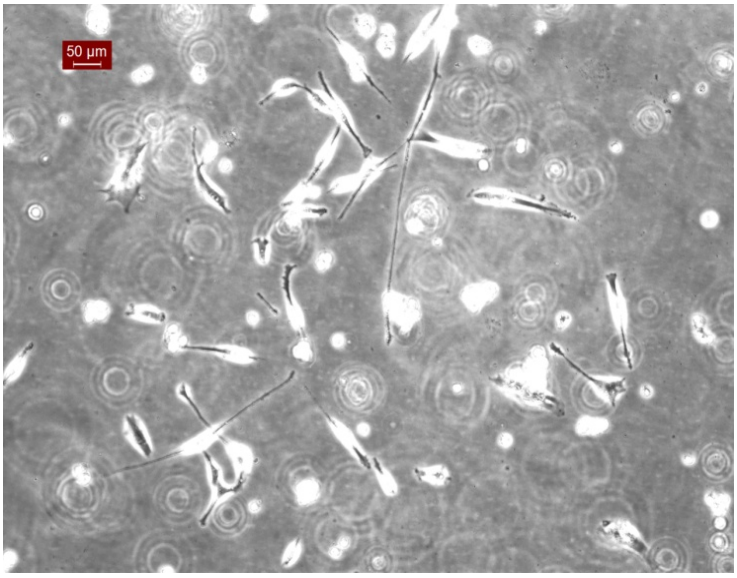


Figure 12: Dendritic cells attached to a T75 flask after 10 days of culture

GATA1 detection and biotinylation efficiency in dendritic cells

GATA1 was undetectable in high salt nuclear extracts of dendritic cells when GM-SCF was used, because the extracted protein amount. Proteins were concentrated by TCA precipitation and the entire protein sample was loaded on an 8% acrylamide gel. In Figure 13A, GATA1 was detected in nuclear extracts of the BirA control mice but not in whole cell extract. In Figure 13B, a band appears at the molecular weight of GATA1s. This band does not appear in BirA samples suggesting that this band is GATA1s. However it is not a clear result as there is no positive control to be compared.

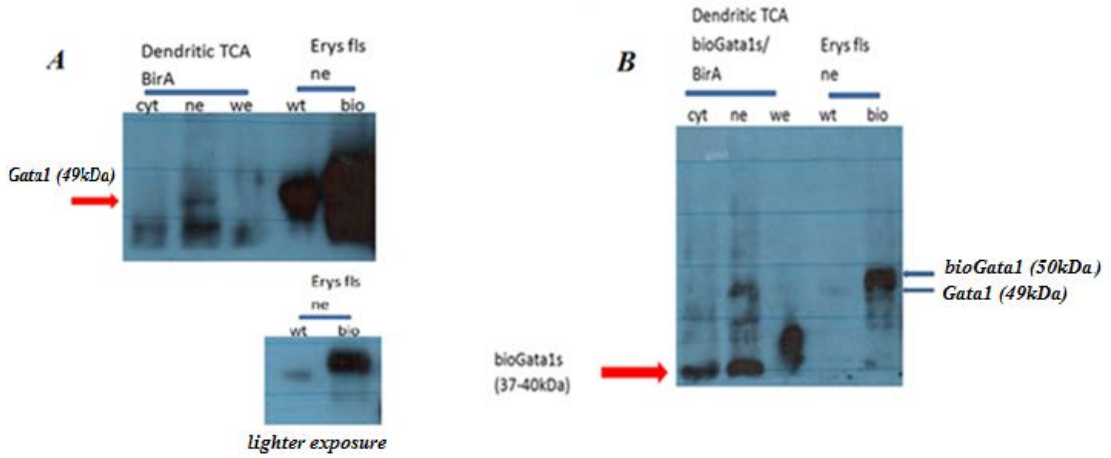


Figure 13: Western blot for GATA1 isoforms after culturing with GMCSF. Erythroid cells generated from fetal livers that express abundantly GATA1 were used as controls. A) Nuclear protein extracts after TCA, from control BirA mice using N6 antibody that interacts with N-terminous of GATA1. B) Nuclear protein extracts after TCA, from bioGATA1short/BirA mice using M20 antibody that interacts with C-terminous of GATA1.

By generating dendritic cells with Flt3, there was higher protein amount in the samples (Table 1), which could be due to the fact that Flt3 stimulates the production of both myeloid and lymphoid DCs (Genetics et al., 1996)(Shurin et al., 1997) (Pulendran et al., 1997). In addition, stimulation with LPS on day 8 of culturing, activates dendritic cells thus promoting them to express more GATA1 (Gutierrez et al., 2007). Regardless, GATA1 immunoprecipitation was carried out in order to ensure GATA1 detection.

As shown in Figure 14A, the IP did not work as there was a smear obscuring detections of a GATA1-specific protein band. However, the input lane revealed that can be detected in the nuclear extracts of control BirA dendritic cells.

In Figure 14B, again a smear appears in the IP samples, but GATA1s was detected in the input as compared with bioGATA1s in the controlsample from fetal liver erythroid cells.

Both BirA and bioGATA1s membranes, were stripped and then incubated with anti-HA antibody. In Figures 14C and 14D, BirA can be detected even though it was undetectable in the positive fetal liver control because of low protein amount.

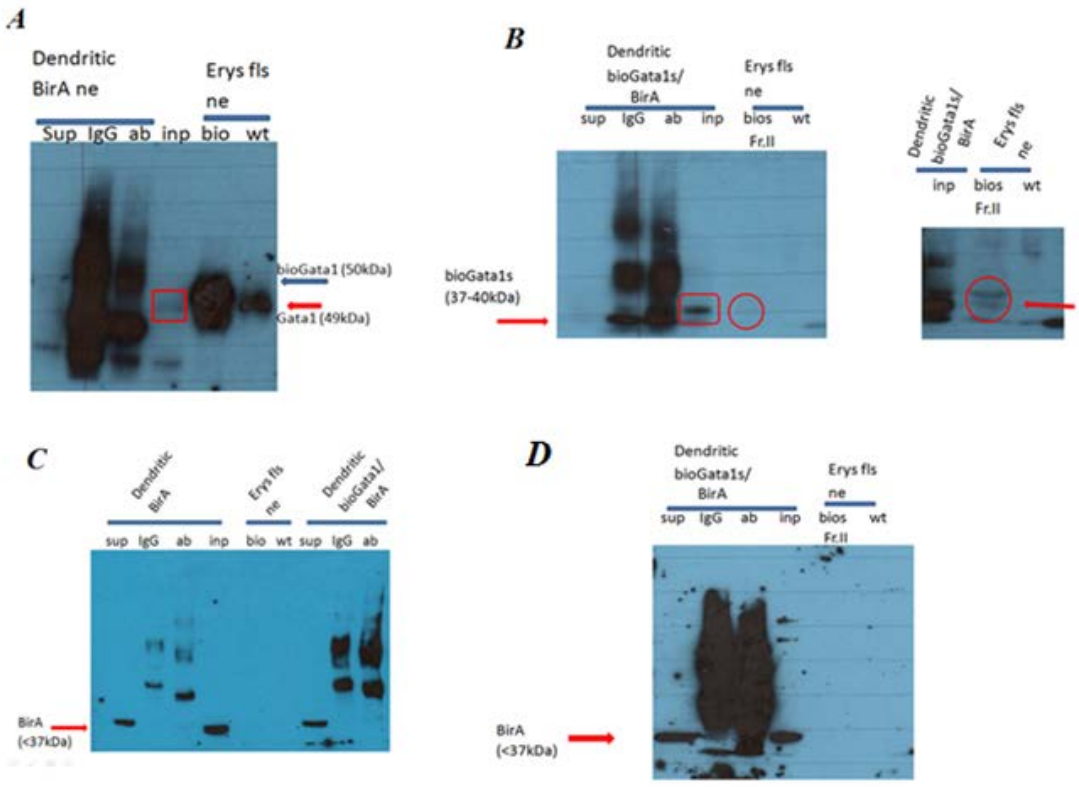


Figure 14: Western blot for GATA1 isoforms after culturing with Flt3. Erythroid cells generated from fetal livers that express abundantly GATA1 or GATA1short were used as controls. A) Nuclear protein extracts after IP for GATA1, from control BirA mice. B) Nuclear protein extracts after IP for GATA1short, from bioGATA1short/BirA mice. C) BirA protein detection in BirA and bioGATA1/BirA mice after stripping IP membranes. D) BirA protein detection in bioGATA1short/BirA mice after stripping IP membranes.

In summary, dendritic cells were optimally generated when expanded by bone marrow cells in the presence of Flt-3. LPS stimulation increased GATA1 expression suggesting that Flt-3 protocol is preferable for GATA1 detection and isolation in dendritic cells.

4.4 Mast cells

Mast cells verification

Mast cells need IL-3 cytokine for their growth and survival. More specifically, Stat5 which is activated downstream of the IL-3 pathway, seems to be essential for Bcl-X_L and Bcl-2 anti-apoptotic protein activation(Shelburne et al., 2012)(Yamasaki et al., 2016). The latter two proteins induce the binding of HC (highly cytokinergic) and PC (poorly cytokinergic) IgEs to FcεRI which is the main receptor for mast cell growth and survival (Rivera & Gilfillan, 2006).

We attempted to generate, mast cells from adult mouse bone marrow. Mast cell generation was induced by using 30ng/ml IL-3. The culture was maintained at 10^6c/ml and medium was changed twice a week. On week 4 the only cells in suspension were mast cells characterized by their small round size (Figure 15). The cells were harvested and washed 2-3 times with PBS (Ishijima, Ohmori, Uenishi, & Ohneda, 2012b) followed by staining with with May-Grunwald. Their staining pattern and their small size suggested that the cells produced upon IL-3 culture of bon marrow cells are mast cells.

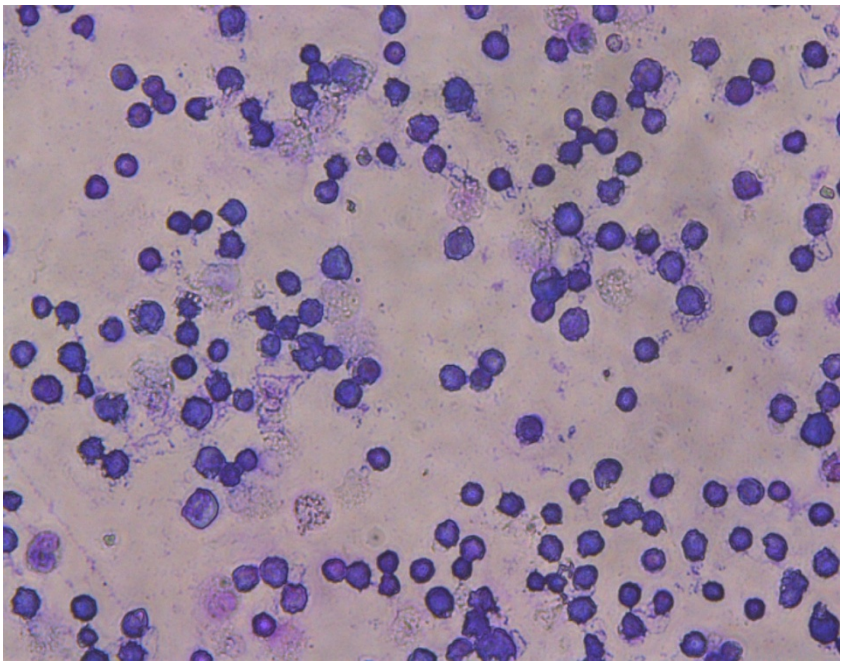


Figure 15: Mast cells after May-Grunwald staining.

We can thus proceed to investigate GATA1 expression in mast cells.

GATA1 detection and biotinylation detection in mast cells

Mast cells express GATA1 and GATA1short at low levels. It has been established that GATA1 regulates mast cell activation but not their maturation (Yamamoto et al., 2014). GATA1 could not be detected in mast cells, unless bone marrow progenitors are cultured in the presence of both IL-3 and SCF (stem cell factor) (Harigae et al., 1998).

Considering the observations mentioned above, we proceeded to immunoprecipitation experiments for GATA1 detection in mast cells.

In Figure 16A, whole cell protein extracts from bone marrow derived mast cells were tested for GATA1 by Western blot. In the BirA immunoprecipitate sample a band of the correct size is detected when probed with an anti-GATA1 antibody. However, the smear visible in the IgG sample made it difficult to distinguish if this was a clear band of GATA1 instead of background.

The same result was observed in Figure 16B, where the bioGATA1/BirA immunoprecipitate sample could not be validated because of the observed smear in the IgG control sample.

In Figure 16C, an expected GATA1s size band of bioGATA1s/BirA is appeared while there is no background at the IgG control. However, there is no positive control to compare it.

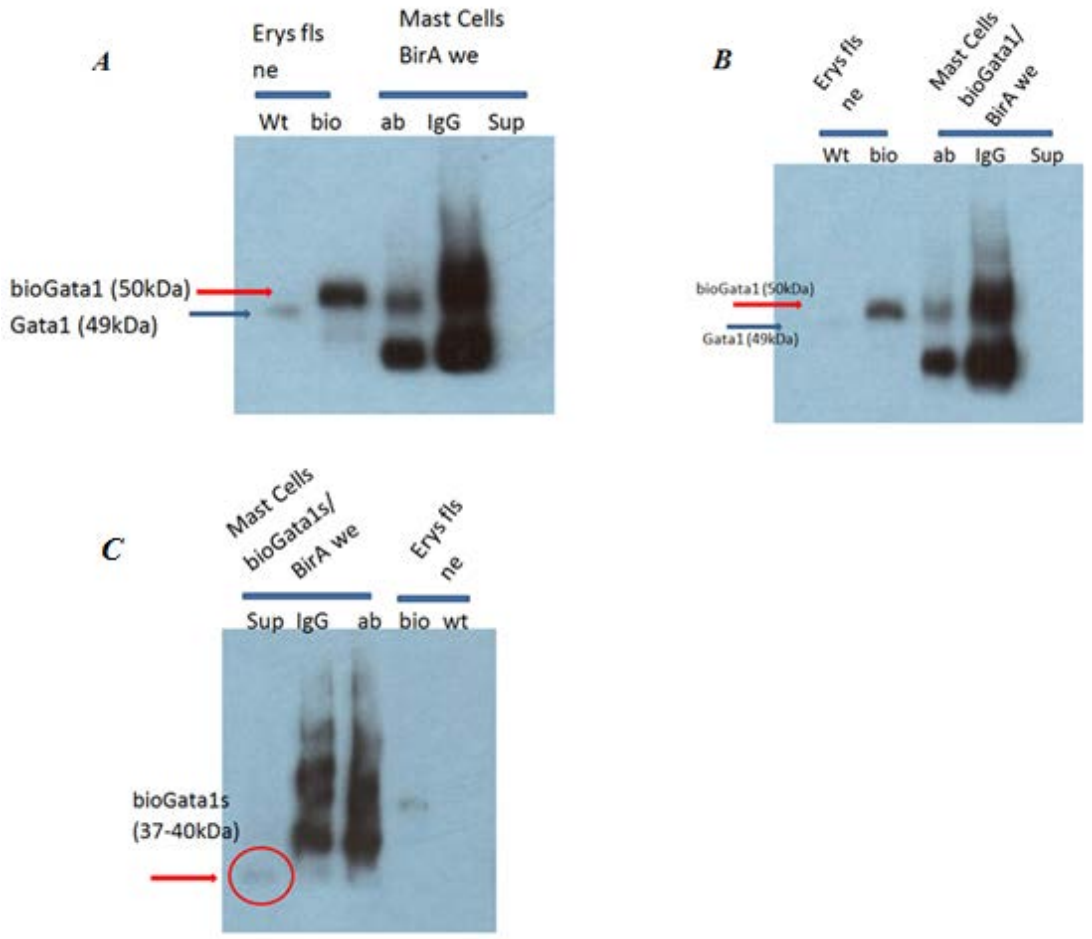


Figure 16: Western blot of mast cells cultured with IL-3.Erythroid cells generated from fetal livers that express abundantly GATA1 or GATA1short were used as controls. A) IP for GATA1 using N6 antobidy, in whole cell extracts of BirA mice. B)IP for GATA1 using N6 antobidy, in whole cell extracts of bioGATA1/BirA mice. C) IP for GATA1 using N6 antobidy, in whole cell extracts of bioGATA1short/BirA mice.

In summary, it appears that mast cells produce low levels of GATA1, especially when IL-3 and SCF are absent (Table 1). Taken together this preliminary evidence suggests that characterization of GATA1 complexes analysis in mast cells may not be feasible.

Table 1: The average protein amount extracted from each lineage in accordance to the isolated number of cells.

	Number of cells	**Nuclear protein extracted**
Megakaryocytes	$2x10^6$/7 fetal livers	25-30µg
Eosinophils	10^8/ a large spleen	100µg
Dendritic cells	1. GM-SCF :10^7/ mouse 2. Flt-3 : 10^6/ 2 mice	54 µg 120µg
Mast cells	$2x10^7$/2 mice	65µg

5. DISCUSSION

In an effort to assess the utility of biotin tagged GATA1 or GATA1s knock-in mouse models in investigating GATA1 functions in megakaryocytes, eosinophils, dendritic

cells and mast cells, we performed isolation and *in vitro* culturing on the aforementioned hematopoietic lineages, followed by the detection of GATA1 or GATA1s, BirA expression and of GATA1 biotinylation efficiency in these lineages. The results are discussed below.

Megakaryocytes

It has long been established that megakaryocyte-specific genes contain GATA1-specific binding sites in their promoters (Evans et al., 1988)(Martin & Orkin, 1990)(Wall & Grosveld, 1988)(Shirihai et al., 2000) (Lemarchandel et al., 1993)(Deveaux et al., 2016)(Ravid et al., 1991)(Hickey & Roths, 1993)(Yagi et al., 1994).. Further experiments on GATA1 null megakaryocytes revealed that the absence of GATA1 was connected to the decrease of megakaryocyte-specific genes expression and to impaired 2N ploidy (P. Vyas et al., 1999). In addition, the overexpression of GATA1 in megakaryocytes resulted in an increase on megakaryocytic differentiation (Kulessa et al., 1995).

In this study, megakaryocytes were isolated from E12.5 fetal livers and successfully verified using May-Grunwald staining and Western blot for Fli-1 as an example of a megakaryocyte-specific transcription factor. GATA1 expression was successfully detected in wild type megakaryocytes, whereas BirA expression was also detected in BirA and bioGATA1s/BirA megakaryocytes. BioGATA1 was undetectable because of the low protein quantity that was available in this experiment. Biotinylation efficiency was tested only in bioGATA1s/BirA samples where a detectable band suggested that bioGATA1s is biotinylated fetal liver derived megakaryocytes. On the basis of these experiments it can be reasoned that full length bioGATA1 is most likely to also be biotinylated, pending experimental verification. In addition, taking into account the fact that 7 fetal livers provided a yield of 25-30μg of nuclear extract, it should be feasible to carry out Mass Spectrometry Analysis to reveal GATA1 and GATA1s protein complexes during megakaryopoiesis.

Eosinophils

The first evidence of GATA1 implication in eosinophil formation was that the expression of GATA1 in myb-ETS-transformed chicken myeloblasts induced their

reprogramming into eosinophils (Kulessa et al., 1995). Consequently, GATA1 binding sites were found to be present in the promoters of eosinophil-specific genes (Du et al., 2002). When GATA1 was knocked out, it resulted in the depletion of the eosinophilic lineage (Yu et al., 2002). Other studies demonstrated that PU.1 and GATA1 are cooperating in eosinophilic lineage regulation (Du et al., 2002).

In this study, LD Column stringent depletion was performed to isolate eosinophils in sufficient quantities from the dissected spleen of an IL-5 transgenic mouse presenting with eosinophilia. The starting cell number was 10^9 cells from an enlarged spleen, ending up with 10^8 eosinophils that gave a yield of approximately 100μg of nuclear extract. BioGATA1 could only be detected after GATA1-IP, whereas BirA was undetectable. Noticeably, this technique could be improved only by using more beads per column during eosinophils isolation, and higher protein amount for the IP experiment. Otherwise, GATA1 protein complex purification form eosinophilsand Mass Spectrometry Analysis will not be feasible.

Dendritic cells

GATA1 is detected in both the cytoplasm and nucleus of Dendritic cells (DCs) (Kozma et al., 2010). GATA1 appears to determine the cell fate between mast cells and dendritic cells. It has been proposed that GATA1 antagonizes and represses the PU.1 interaction with its gene targets, suppressing dendritic cell formation (Shimokawa et al., 2010). Moreover, GATA1 is also found to be essential in the activation of DCs, after LPS stimulation, when GATA1 is found to be expressed in all DC precursors and in all differentiated DC subtypes and is vital for their survival (Gutie et al., 2016).

In the present study, DC precursors were isolated from the bone marrow of adult mice and were cultured in the presence of either Flt-3 or GM-SCF. In both protocols dendritic cells were successfully verified at the end of culture by the recognition of their branched morphology. The Flt-3 protocol gave better results as it provided us with 10^6cells and 120μg of nuclear extract per mouse in contrast with the GM-SCF protocol which resulted in 10^7cells and 54μg of nuclear extract. The Flt-3 protocol made feasible the detection of bioGATA1 and bioGATA1s isoforms. In addition, expression of BirA could also be clearly detected in both sample. Biotinylation could not be verified using streptavidin-HRP, as proteins were not detectable in the western

blot membranes after the second stripping. All in all, Flt3 protocol provided us with sufficient quantity and better quality of nuclear extracts in order to detect bioGATA1, bioGATA1s and BirA without IP. Hence, this protocol holds promise for proceeding to Mass Spectrometry and for GATA1 complex characterization using DCs isolated from the bone marrow of 10 mice.

Mast cells

The first the connection between GATA1 and mast cells was the observation that GATA binding sites were found in the regulatory regions of genes specifically expressed in mast cells (Nishiyama et al., 2016)(L. Zon et al., 1991)(Yatsunamilg et al., 1994)(Caugheys et al., 1991)(Kwan et al., 2005). However, GATA1 is undetectable in non-activated mast cells (Harigae et al., 1998). Notably, bone marrow mast cells express GATA1 only after addition of stem cell Factor and IL-3, which are cytokines that provoke mast cell activation (Harigae et al., 1998). It has been suggested that GATA1 is essential only for MC degranulation as GATA1 ablation affected only MC activation but not their formation (Yamamoto et al., 2014)

In an effort to detect GATA1 in mast cells, we successfully verified bone marrow derived mast cells cultured in the presence of IL-3 but no SCF using May-Grunwald staining. These cells were found to express GATA1, bioGATA1 and probably GATA1s only after GATA1-immunoprecipitation. BirA expression was undetectable. Their small size agrees with the fact that we obtained $2x10^7$ cells per 2 mice that yielded approximately 65μg of nuclear extracts.

In the present study we found that megakaryocytes and dendritic cells express GATA1 in such levels that it can be detected without immunoprecipitation. BirA detection also suggests that bioGATA1 and bioGATA1s are in vivo biotinylated in these lineages. However, further streptavidin Western blots and pull downs, need to be carried out to verify biotinylation. Eosinophils were also found to express detectable bioGATA1, but we did not manage to clearly detect BirA expression or biotinylation. Mast cells do not require GATA1 for their formation as GATA1 is detectable only in activated MCs when Il-3 is present. Taken together our results suggest that megakaryocytes, dendritic cells and eosinophils represent hematopoietic

lineages that could be used for GATA1 protein complex characterization using *in vivo* biotinylation tagging. Mass Spectrometric analysis will reveal GATA1 protein complexes during the differentiation of the aforementioned hematopoietic lineages and may thus shed light in the GATA1 role during hematopoiesis and open up new therapeutic approaches in treating hematopoietic diseases.

More specifically, the GATA1s isoform that lacks the N-terminal domain (Rainis et al., 2003) (Calligaris et al., 1995) has been linked to Diamond Blackfan Anemia (DBA) (Parrella et al., 2015) and transient myeloproliferative disease (TMD/TL) (Maroz et al., 2013) or acute megakaryoblastic leukemia (AMKL) which occur with high frequency in Down Syndrome (DS) patients. It appears that GATA1s can recognize and bind to megakaryocyte-specific genes but binding to erythroid genes is negatively affected (Albuquerque et al., 2006). In addition, GATA1s has no affinity for binding to RB which is essential for erythropoiesis (Kadri et al., 2009). In DS transient leukemia patients, GATA1s seems to promote eosinophils accumulation (Maroz et al., 2013). Hence, the differential characterization of protein complexes formed by full length GATA1 versus GATA1s in these hematopoietic lineages using the biotin tag GATA1 knock-in mouse models will help shed light into the implication of GATA1 s in the pathogenesis of these hematological diseases.

In summary, the present study suggests that biotin tagged GATA1 and GATA1s mice will help to understand the contribution of GATA1 and GATA1s protein in hematopoiesis and will permit diagnostic and therapeutic advances in the hematopoietic diseases to which they are related.

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