Thèse présentée par :

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Pour obtenir le grade de : *Docteur de l'université de Strasbourg*

Sciences du Vivant

Aspects Moléculaires et Cellulaires de la Biologie

Studying the posttranslational modifications of transcription factor Ikaros and their role in its function.

Soutenue le : **28 septembre 2012 devant le jury composé de:**

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Rapporteur externe :	**Dr. Angelita REBOLLO**
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Acknowledgements

I am deeply grateful to my PhD supervisors: Dr. Susan CHAN and Dr. Philippe KASTNER for offering me the opportunity to work and learn in this laboratory. They were patient, forgiving and able to understand the difficulties, which I had to overcome during the last three years.

I want to give a special thanks to Attila ORAVECZ who was helping me throughout my PhD study, by sharing his experimental knowledge and devoting a lot of time in helpful discussions with me.

I wish to thank to my colleague and friend Jérôme MASTIO for his priceless help during both my Master and PhD degree, providing me with lectures and all kind of help that I needed.

I also wish to thank to all the other members of our laboratory for their support and understanding, while working on the challenging field of sumoylation. In addition to the scientific part, I spent very funny moments with you, which I will never forget.

Many thanks to the members of Bernardo REINA-SAN-MARTIN's lab, which are always open for discussions and ready to help.

I would like to thank to my family for their unconditional love and support since I have been born.

Last but not least, I would like to thank Dr. Daniel METZGER, Dr. François MORLE and Dr. Angelita REBOLLO for agreeing to read my thesis and judge my work.

Contents

CHAPTER 1

Introduction

I. Kruppel-like family of transcription factors

Ikaros family belongs to the larger family of Kruppel-like transcription factors (KLF), which contains at least 17 members that play an important role in differentiation, function, and homeostasis of many cell types. Its members have 3 contiguous C2H2-type zinc fingers at the carboxyl terminus that comprise the DNA-binding domain with consensus amino acid signature ([Y/F] xCx2Cx3Fx5Lx2HxRxHTGEKP) that recognizes CACCC consensus DNA motif (Suzuki et al. 2005). In addition to their DNA binding module, the KLF family members contain domains that activate or repress transcription (Cao et al., 2010). They regulate the expression of genes that have GC-rich promoters and modulate different cellular functions including cell proliferation, differentiation, apoptosis or malignancies such as neoplastic transformation. Members of this family can function as repressors or activators depending on the promoter context and their interacting partners (Kaczynski et al. 2003). A number of these factors show developmental and pathological implications in the vasculature (Suzuki et al. 2005) and importance in the biochemistry of neuronal differentiation (Seo et al. 2012). These proteins are also involved in the lymphocyte differentiation. Kruppel-like factor 4 (KLF4) promotes the survival of natural killer cells and maintains the number of conventional dendritic cells in the spleen (Park et al. 2012) while KLF1 also known as EKLF1 is critical for the switch to adult hemoglobin expression in developing erythrocytes (Hart et al. 2012). The development and maturation of another important T-cell subtype such as Tregs depends on KLF10. Its deficiency leads to reduced differentiation of Foxp3+CD4+ T cells and decreased ability of these cells to suppress airway inflammation in vivo (Venuprasad et al. 2008).

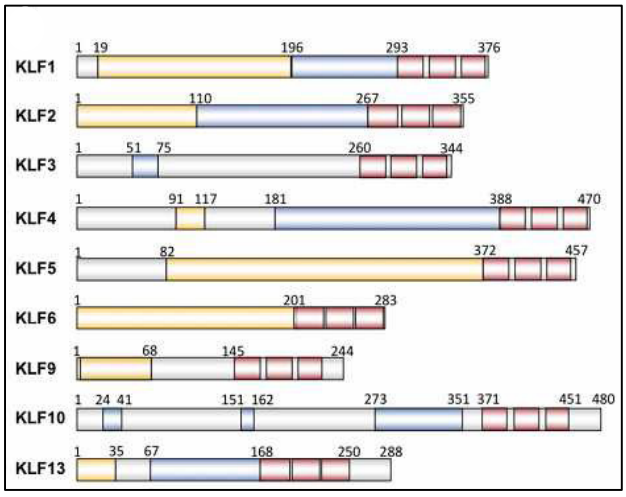


Figure 1. Schematic representation of the different functional domains of leukocyte-associated KLFs.

The DNA-binding "zinc fingers" are represented by red boxes. The trans-activation domains are indicated by orange boxes, whereas the trans-repression domains are indicated by blue boxes. Adapted from (Cao et al., 2010).

I. 1. Ikaros family

Ikaros is the founding member of the Ikaros family of transcription factors that belongs to the larger - Kruppel-like family. It was first described as a zinc finger containing protein that regulates the differentiation of the T cell lineage (Georgopoulos al., 1992). Up to now five Ikaros family members are known - Ikaros, Helios, Aiolos, Eos and Pegasus (Fig 2).

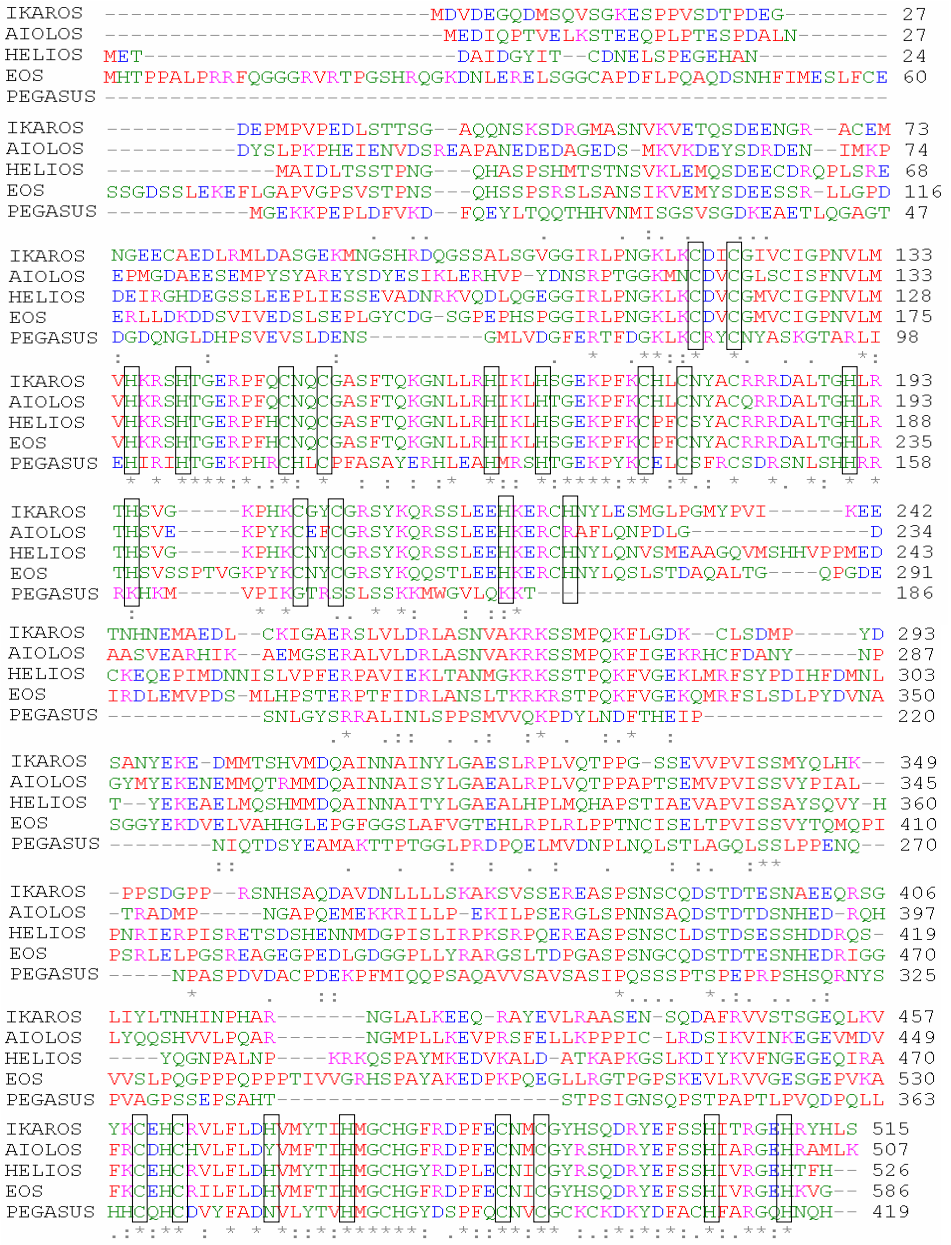


Figure 2. Alignement of the sequence of Ikaros family members (mouse).

The amino acids are indicated with different colors that correspond to their biochemical properties.

Blue (negatively charged)

Pink (positively charged)

Green (uncharged)

Red (hydrophobic)

The open boxes show the alignment of the Cys and His residues that shape the DNA binding and dimerization Zn Finger domains.

Human and mouse Ikaros share 95% overall identity (Molnar et al., 1996)

I. 2. Ikaros family structure and mechanism of function as transcription factors

Ikaros family members contain six C_2H_2 zinc finger domains: four N-terminal - involved in DNA binding and two C-terminal required for homo and hetero dimerization, which is also essential for the stability of these proteins (Figure 3) (Molnar and Georgopoulos, 1994). The more detailed description of Ikaros exons is shown on figure 7-A.

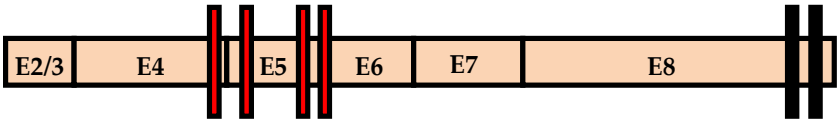


Figure 3. Schematic representation of Ikaros and its functional domains.

The exons E2/3 to E7 are indicated as pink horizontal rectangles (E1 is non-coding). The four zinc fingers that shape the DNA-binding domain are shown by red vertical rectangles. The two zinc fingers that define the dimerization domain are shown by black vertical rectangles. Adapted from (Koipally and Georgopoulos, 2002).

The DNA binding domain of Ikaros members is homologous among all the members except Pegasus, which lacks the first N-terminal zinc finger (Pedromo et al., 2000). Expectedly, all the Ikaros family members are able to recognize the same consensus motif TGGGAA, except Pegasus, which has been reported to bind the unrelated GxxxGxxG sequence. Fusion proteins containing the N-terminal part of Ikaros are able to bind the same sequences as the wild type Ikaros, while isoforms containing less than three zinc fingers don't efficiently bind the target DNA (Hahm et al., 1998; Morgan et al., 1997; Molnar and Georgopoulos, 1994; Morgan et al., 1997; Pedromo et al., 2000). The nuclear targeting of Ikaros is due to the presence of non-classical nuclear localization signal (NLS) across fingers two and three in the DNA-binding domain. The deletion of these parts results in complete loss of nuclear targeting, a phenotype rescued by the addition of heterologous NLS to the mutants (Cobb et al., 2000).

Unlike the N-terminal DNA binding domain, all the family members, including Pegasus share homologous C-terminal dimerization domain, which confers them the ability to homo- and hetero- dimerize. (Sun et al., 1996; McCarty et al., 2003; Hahm et al., 1998; Morgan et al., 1997; Pedromo et al., 2000). The ability of Ikaros to dimerize is crucial for its functions, because the mutations that impair the dimerization also abolish its DNA-binding properties, as well as its ability to regulate transcription. Homo- and hetero- dimerization between isoforms that contain four functional DNA-binding zinc fingers greatly increases both their affinity for DNA and their transcriptional activity (Sun et al., 1996). Dimers originating from isoforms, in which more than one DNA-binding zinc fingers is missing, are associated with aberrant hematopoiesis and malignancies, such as leukemia (Georgopoulos et al., 1994; Rebollo and Schmitt, 2003; Zhang et al., 2007; Winandy et al., 1995).

I. 3. Accepted functional models of transcriptional regulation by Ikaros

In addition to the DNA-binding and dimerization domains, all Ikaros proteins contain a bipartite activation region located in proximity to their dimerization domain (Sun et al., 1996). Through this domain Ikaros, Helios and Aiolos are able to activate transcription of reporter genes driven by the Ikaros consensus binding site, GGGAA (Molnar and Georgopoulos, 1994; Kelley et al., 1998). It was discovered using the yeast one-hybrid system and GAL-4 transactivation assay in mammalian cells. Using deletional analysis, this domain was shown to be within the first 81 amino acids encoded by exon 7 and is typical for all of the Ikaros isoforms. Inspection of this region revealed a stretch of both acidic and hydrophobic residues, as the last ones have been found to be required for maximal activity of this activation region. Interestingly, the hydrophobic amino acid stretch alone was unable to act as an activator of transcription (Sun et al., 1996).

The Ikaros functional regions described above are also protein-binding determinants, able to interact with proteins belonging to different chromatin remodeling complexes, as well as with co-repressors. These interactions are crucial for Ikaros function as activator or repressor of the transcription (Figure 4).

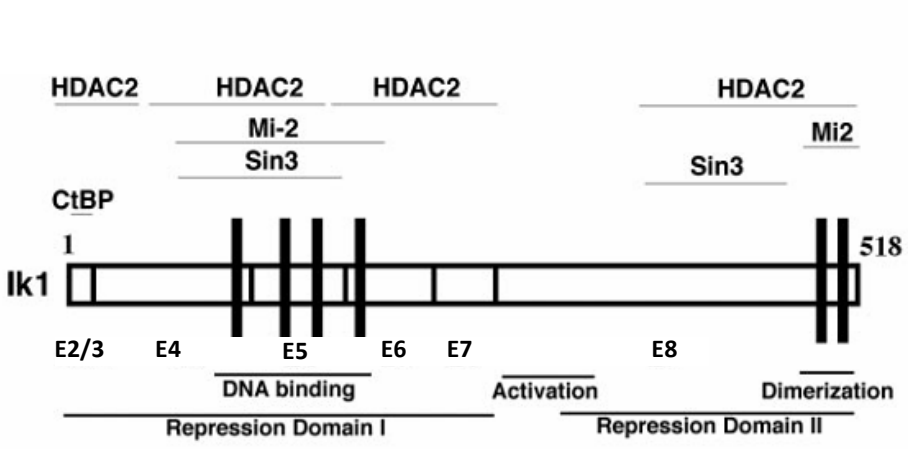


Figure 4. Schematic representation of Ikaros and its functional domains.

The exons (E2/3 to E8) are indicated as open horizontal rectangles. The zinc fingers are shown by black vertical rectangles. The parts of the protein that are involved in the interaction with different partners are indicated as grey lines. Adapted from (Koipally and Georgopoulos, 2002).

I. 4. Ikaros as an activator of the transcription

It has been reported that Ikaros contains an activation domain that is able to stimulate transcription in reporter gene assays in non-lymphoid cells. More likely, Ikaros enhances the gene expression by helping bona fide activators like Sp1, rather than to promote a direct transcriptional activation. One of the possible explanations for this phenomenon of up-regulated gene expression is co-repressor squelching rather than direct recruitment of the co-activators (Koipally and Georgopoulos, 2002). Moreover, the other members of Ikaros family Helios and Aiolos are also able to potentiate transcription in ectopic transfection assays (Kelley et al., 1998; Molnar and Georgopoulos 1994; Morgan et al., 1997) or in muscle or neural cells for Eos (Bao et al., 2004; Yeung et al., 2012). It has been shown that Ikaros functions as an activator of the CD8α and STAT4 genes, events important for thymocyte differentiation and lineage commitment (Harker et al., 2002; Yap et al., 2005). The transcription activation function of Ikaros has been linked to its interaction with potentiating transcription chromatin remodeling complexes. In the T-cell nucleus, a small fraction of Ikaros and Aiolos proteins has been found to associate with Brg-1, a component of the SWI/SNF complex (Kim et al., 1999). An activating role of Ikaros as a potentiator of transcription initiation and elongation has been described in erythroid cells. In this case Ikaros interacts with the cyclin-dependent kinase P-TEFb (positive transcription elongation

factor b) and cooperates with GATA-1 to enhance transcription of the human γ-globin promoter (Bottardi et al., 2011). In addition, cooperation of Ikaros with another factor of the same family - GATA-3 increases IL-10 production in CD4+ T cells. In this case Ikaros directly binds to the conserved regulatory region of the Il10 gene locus in Th2 cells, suggesting a direct role of Ikaros in Il10 transcriptional regulation (Umetsu and Winandy, 2009).

I. 5. Ikaros as a transcriptional repressor

Despite the fact that Ikaros family members are able to potentiate transcription, their main role has been suggested to negatively regulate the transcription of their target genes. All the members of Ikaros family are able to repress transcription of endogenous or reporter gene transcription (Hu et al., 2007; Koipally et al., 1999; Pedromo et al., 2000; Tabayashi et al., 2007).

There are several different described ways by which Ikaros can act as a transcriptional repressor. One of them is the ability to recruit silencing chromatin remodeling complexes, such as NURD (Nucleosome Remodeling and Deacetylase Repressor Complex) at the promoters of its direct target genes. This complex contains not only ATP-dependent nucleosome disruption activity conferred by Mi-2α, Mi-2β subunits, but also histone deacetylase activity of HDAC-1 and 2, which usually associates with transcriptional repression. The deacetylation is stimulated by ATP on nucleosomal templates, suggesting that nucleosome disruption aids the deacetylase to access its substrates (Xue et al., 1998). In fact, in addition to Ikaros, Helios, Aiolos and Eos are also found to interact both *in vitro* and in primary cells with NURD components, such as Mi-2α, Mi-2β, HDAC-1/2 and MTA-1/2, as well as with co-repressors, such as Sin3A/Sin3B (Hu et al., 2007; Kim et al., 1999; Koipally et al., 1999; Sridharan and Smale, 2007; Koipally and Georgopoulos, 2002a). Ikaros, Helios and Eos are also able to repress transcription by interacting with co-repressors, in a HDAC independent manner. These family members interact with C-terminal binding protein CtBP and CtBP-interacting protein –CtIP, leading to HDAC independent transcriptional repression (Figure 5)(Hu et al., 2007; Koipally and Georgopoulos 2002a; Pedromo and Crossley 2002).

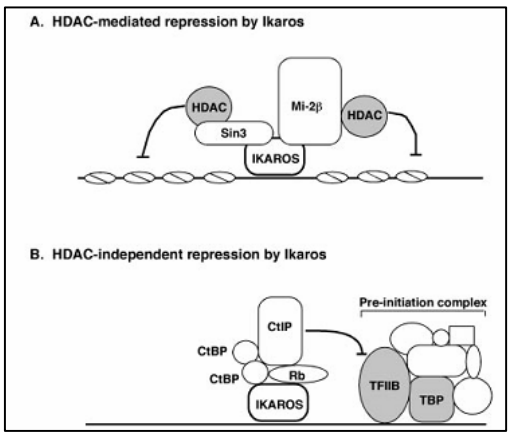


Figure 5. Model showing different repression strategies of Ikaros.

(A). Ikaros recruits HDACs through Mi-2β and/or Sin3 proteins to a promoter, resulting in compact chromatin configuration, which restricts the transcription. (B). Ikaros recruits CtBP, CtIP, and Rb to a promoter, all of which can interact with components of the general transcriptional machinery, thus negatively modulating the transcription initiation. Adapted from (Koipally and Georgopoulos 2002a)

Another possible mechanism of Ikaros mediated transcriptional repression lies on its abilities to recruit its target genes to transcriptionally inactive pericentromeric heterochromatin loci. Ikaros shows punctuate distribution pattern in the nucleus of resting T-cells (Hahm et al., 1998; Kelley et al., 1998; Morgan et al., 1997). Upon activation, Ikaros localizes to the heterochromatin-associated toroids, a domain in which a number of transcriptionally silent genes are localized (Brown et al., 1997). This distribution is dependent on the presence of an intact DNA-binding domain, which binds pericentromeric repeated sequences, thus recruiting the target gene to the loci (Cobb et al., 2000). This working model is based on the assumption that Ikaros dimers bound to their target are able to oligomerize with other dimers bound to the pericentromeric DNA sequences, thus localizing the targets gene in transcriptionally inactive zones of the chromatin (Trinh et al., 2001). Interestingly, Ikaros can silence transcription by recruiting genes to heterochromatin, without changing the methylation status of their promoters. Localized to pericentromeric heterochromatin Ikaros is able to initiate silencing of λ5 gene expression through a direct effect on its promoter rather than causing its heterochromatinization by spreading methylation marks (Sabbattini et al., 2001).

Ikaros can also repress transcription by competing with transcription factors up-regulating the gene expression, occupying a target site also used by the transcriptional activator. Examples for such a competitive couples are Ikaros-RBPJκ on the promotor of Hes1 gene (Kleinmann et al.,

2008), Ikaros-EBF (early B-cell factor) on λ5 gene (Thompson et al., 2007) and Ikaros-Elf1 on the terminal deoxynucleotidyl transferase (TdT) (Trinh et al., 2001).

The complexity of Ikaros functions might be outlined by the different mechanisms of Ikaros mediated transcriptional repression, including target gene recruitment to transcriptionally inactive loci, altering the chromatin accessibility by silencing remodeling complexes and direct competition for binding sites with other transcription factors.

The current understanding of how Ikaros regulates transcription includes not only the targeting of the NuRD complex but also modulating its activity in lymphocytes, thus influencing developmental gene-expression programs (Figure 6)(Oestreich and Weinmann, 2012).

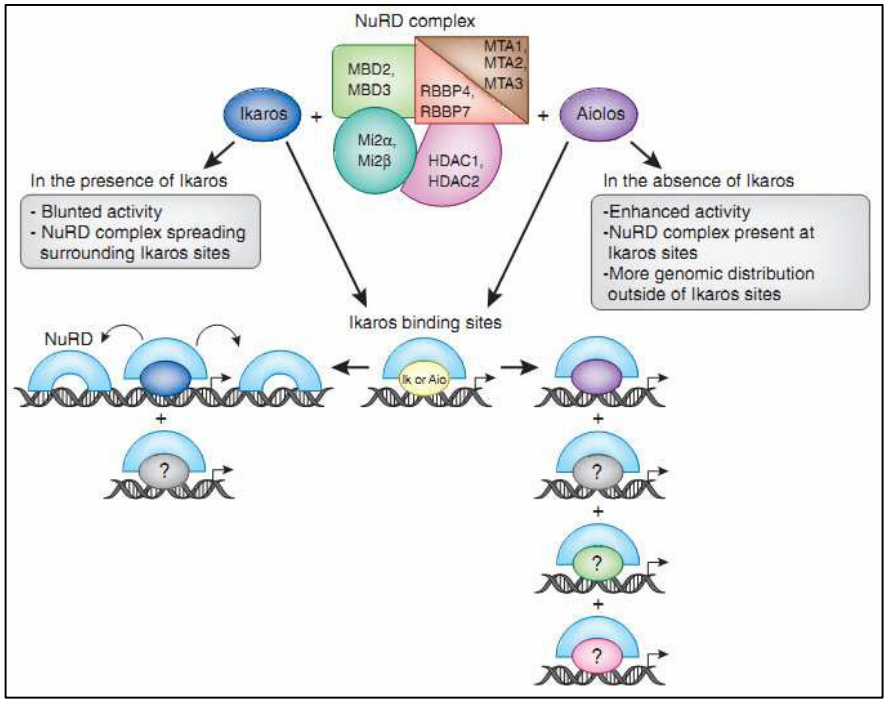


Figure 6. Ikaros influences both the genomic localization and functional activity of the Mi-2β NuRD complex.

In lymphocytes Ikaros and Aiolos associate with the NURD complex and target it to Ikaros or Aiolos DNA-binding sites. If the NuRD complex is targeted to loci in the presence of Ikaros, the NuRD complex spreads to adjacent regions but, interestingly, the activity of the complex located in these regions is blunted. In contrast, in the absence of Ikaros, the NuRD complexes targeted by Aiolos can no longer spread in the region, but these complexes have enhanced activity. Surprisingly, in cells that lack Ikaros, the NuRD complex is targeted to many additional genomic regions, independent of the Ikaros or Aiolos DNA-binding elements, and these complexes have enhanced activity at these locations. HDAC, histone deacetylase; MTA, metastasis-associated protein, RBBP, retinoblastoma protein-binding protein. Adapted from (Oestreich and Weinmann, 2012).

I. 6. Ikaros deficient mouse models

I.6.1 Ikaros null mutation ($IK^{-/-}$) (Wang et al., 1996)

The mice of this model express unstable and quickly degraded Ikaros protein resulting in loss of function phenotype, due to the deletion of the C-terminal dimerization domain situated in exon 7. The number of the hematopoietic stem cells in this model is reduced about 30-40 fold, there are no fetal T, γδT cells, but T-cells are able to develop in the post-natal period. There is an appearance of clonal or oligoclonal T cell tumors and absence of $CD8^{-}$DC and reduced $CD8^{+}$DC dendritic cells (Wu et al., 1997). The absence of Ikaros also biases positively the myeloid and negatively the erythroid development with appearance of anemia and delayed globin switching (Nichogiannopoulou et al., 1999). These mice don't have B and NK cells and their precursors.

I.6.2 Dominant negative Ikaros mutation ($IK^{DN/DN}$) (Georgopoulos et al., 1994)

The mice of this model express shorter Ikaros isoform that lacks the exons 3 and 4, resulting in loss of the DNA binding ability. This isoform acts as a dominant negative protein because it is able to dimerize with full length Ikaros family proteins, thus abolishing their DNA binding. These mice have a significantly reduced HSC compartment (more than 100 fold) and there are no T, B or NK cells, as well as their progenitors. 95 % of them die 1 to 3 weeks after birth. The more severe phenotype of this mutation suggests the existence of other members of the same family that cooperate with Ikaros, which were discovered later.

I.6.3 Hypomorphic Ikaros mutation ($IK^{L/L}$) (Kirstetter et al., 2002)

This mouse model has been created in our lab by insertion of the LacZ reporter gene into exon 3 of the Ikzf1 locus. This insertion causes an alternative splicing and subsequent deletion of the exon 3 coding sequence, resulting in deletion of the same part at protein level. This truncated, shorter Ikaros is still functional but expressed at very low levels (about 10 % of the normal level). Despite its presence, the low Ikaros amount in $IK^{L/L}$ homozygous mice results in no fetal and partial postnatal block of the B cells, no mature pDC (plasmacytoid dendritic cells) as well neutrophil block. In the adults, the T- and B cells are hyperproliferative, resulting in 100 % of T lymphomas at 3-4 months of age and a lifespan of about 8 months.

I.6.4 Ikaros plastic mutation IK-Plstc $^{-/-}$ (Papathanasiou et al., 2003)

This model has been established by the intraperitoneal injection of the mutagene compound ethylnitrosourea. The analysis of the mutated Ikaros locus shows a point mutation in the third DNA-binding zinc finger that impairs its interaction with DNA, resulting in embryonic lethality in the homozygous mice. In the heterozygous mice, the terminal differentiation of erythrocytes and granulocytes is the most affected. They show a bias of the myeloid compartment with an increase of macrophages and blocked lymphoid differentiation.

I. 7. Isoforms of Ikaros family

Ikaros, as well as the other members of the family are presented by several isoforms generated by alternative splicing or by abberant Rag-mediated recombination (Mullighan et al., 2008). These isoforms are expressed in normal cells, or in cells undergoing malignant transformation (Hahm et al., 1998; Liippo et al., 2001; Molnar and Georgopoulos, 1994; Nakase et al., 2002; Meleshko et al., 2008). Up to now, the existence of splicing isoforms of Eos and Pegasus has not been reported. The common feature of all the isoforms is the presence of the C-terminal zinc fingers, through which they can homo- and heterodimerize with other isoforms or Ikaros family members. These isoforms differ in their ability to bind DNA and can therefore be separated in two main groups – DNA binding or nonbinding isoforms. It has been shown that proteins with at least three zinc fingers can efficiently bind DNA, while shorter versions (Ik4 to Ik8) act as dominant negative isoforms upon heterodimerization because they lack DNA binding properties (Georgopoulos et al., 1994). The DNA binding isoforms of Ikaros, Aiolos and Helios are able to bind the Ikaros consensus sequence GGGAA, as the affinity of this binding slightly depends on the sequence that flanks this core motif (Molnar and Georgopoulos, 1994). All the isoforms that don't contain zinc fingers 3 and 4 are nonDNA-binding, but are still able to dimerize with all the other isoforms and family members, thus exerting dominant negative effects on their function (Sun et al., 1996; Tabayashi et al., 2007). The isoforms of Ikaros and its family are schematically presented on Figure 7. Interestingly, Aiolos isoform, Aio-1-5a (Figure 7D) contains an intact DNA binding region but lacks the exon 7, which contains the C-terminal zinc fingers required for homo- and heterodimerization. This isoform is interesting because its C-terminal part ends with a distinct

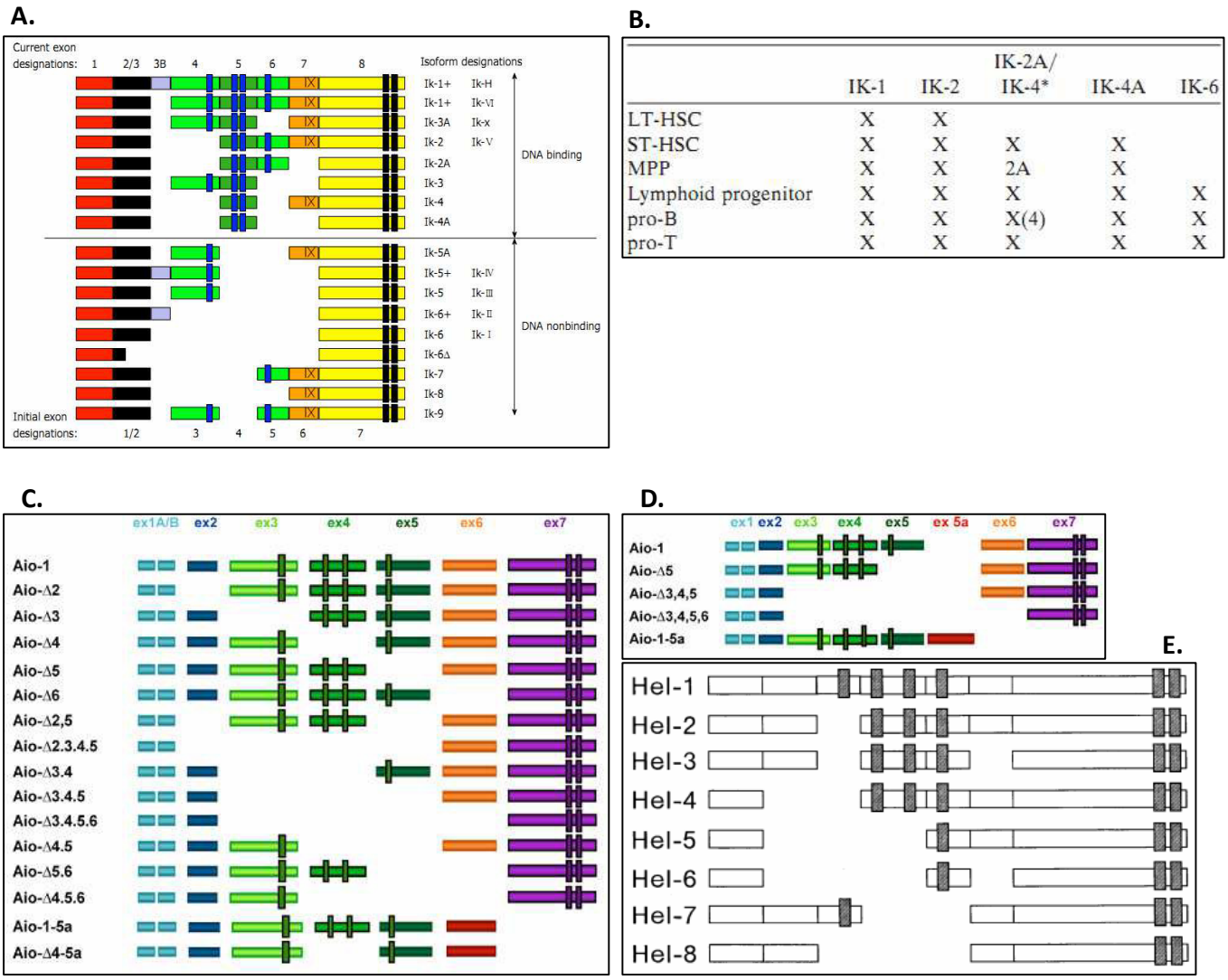


Figure 7. Ikaros family isoforms.

(**A**) Ikaros isoforms. The Ikaros gene in mice (Ikzf1) and humans (IKZF1) includes eight coding exons (exons 2-8 and 3B) and one upstream exon that is not translated (shown in red). This exon has not been identified in initial reports, and the alternate exon designations that have appeared in early reports are shown at the bottom of the figure. Exon 3B is currently not identified as an exon in Genbank. Splice forms that include exon 3B have been designated as "plus" forms and many such splice forms, in addition to the ones shown, have been identified at the protein and/or mRNA level (i.e. Ik-x+, Ik-2+, Ik-4+, Ik-7+, Ik-8+) in humans and mice. An alternate splice site gives rise to splice forms that lack the last 30 bases of exon 7 (indicated with an X). Such splice variants have been designated minus forms (e.g. Ik-1-and Ik-x-). The four N-terminal zinc fingers (shown in blue) contribute to DNA binding and the two C-terminal zinc fingers (shown in black) are responsible for dimerization, adapted from (Francis et al., 2011). (**B**) Ikaros RNA expression in hematopoietic progenitors, adapted from (Klug et al., 1998). (**C,D**) Schematic representation of Aiolos isoforms adapted from (Cabalerro et al., 2007). (**E**) Helios Isoforms, adapted from (Nakase et al., 2002)

Legend concerning (B): * indicates IK-2A/IK-4 co-migrating bands. "X" represents the presence of a band, and 2A or 4 indicates a positive identification verified by sequencing. The Ik-4 has been identified in pro-B cells and found in an Abelson-transformed pre-B cell line, as well as in a T-cell line. All other X designations come from RT-PCR analyses of normal cells sorted by FACS according to their distinctive phenotype.

exon indicated as 5a, but doesn't contain exons 6 and 7. In transient transfection assay in COS-7 cells Aio-1-5a shows both nuclear and cytoplasmic punctuate distribution suggesting an ability of this isoform to dimerize through this alternate exon (Cabalerro et al., 2007).

The most abundant isoforms in primary, non-malignant cells are Ik-1 and Ik-2 for Ikaros, Aio-1 for Aiolos and Hel-1 and Hel-2 for Helios (Molnar and Georgopoulos, 1994; Hahm et al., 1998; Kelley et al., 1998; Caballero et al., 2007).

An interesting observation is the differential expression pattern of Ikaros isoforms during lymphocyte development, suggesting a regulatory role of these variants during normal hematopoiesis (Klug et al., 1998). In the long term hematopoietic stem cells (LT-HSC) compartment, only isoforms Ik-1 and and Ik-2 are detected, while the short term hematopoietic stem cells (ST-HSC) express more Ikaros splice variants (Figure 7-B). The dominant negative (DN) Ik-6 isoform is missing in the HSC compartment but appears during its differentiation into lymphoid and myeloid progenitors.

Different shorter Helios isoforms, such as Hel-5, 6, 7 and 8 are not found to be expressed in cells of healthy donors, but are abundantly expressed in leukemic cells from patients (Nakase et al., 2002; Fujii et al., 2003).

An abberant expression of Ikaros and Aiolos proteins has been detected in leukemic cells. Up-regulated expression of the DN isoform Ik-6 has been reported in childhood and adult Acute Lymphoblasic Leukemia – ALL (Mulligan et al., 2008; Takanashi et al., 2002; Liippo et al., 2001). The expression of a number of dominant-negative and mutant Ikaros isoforms have been reported in infant acute lymphoblastic leukemia (Sun et al., 1999). This study showed, that leukemic cells from infants with ALL express dominant-negative Ikaros isoforms Ik-4, Ik-7, and Ik-8 that lack critical N-terminal zinc fingers. In other group of infant patients, they detected a short in-frame deletion of 10 amino acids of Ik-2, Ik-4, Ik-7, and Ik-8 variants. Interestingly, a single copy or the complete absence of Ikaros was observed in more than 80% of patients with BCR-ABL-positive acute lymphoblastic leukemia (Mullighan et al., 2007) or in patients with acute myelomonocytic and monocytic leukemias, in which it up-regulates the expression of the anti-apoptotic protein Bcl-XL (Yagi et al., 2002).

I. 8. Role of Ikaros during hematopoiesis

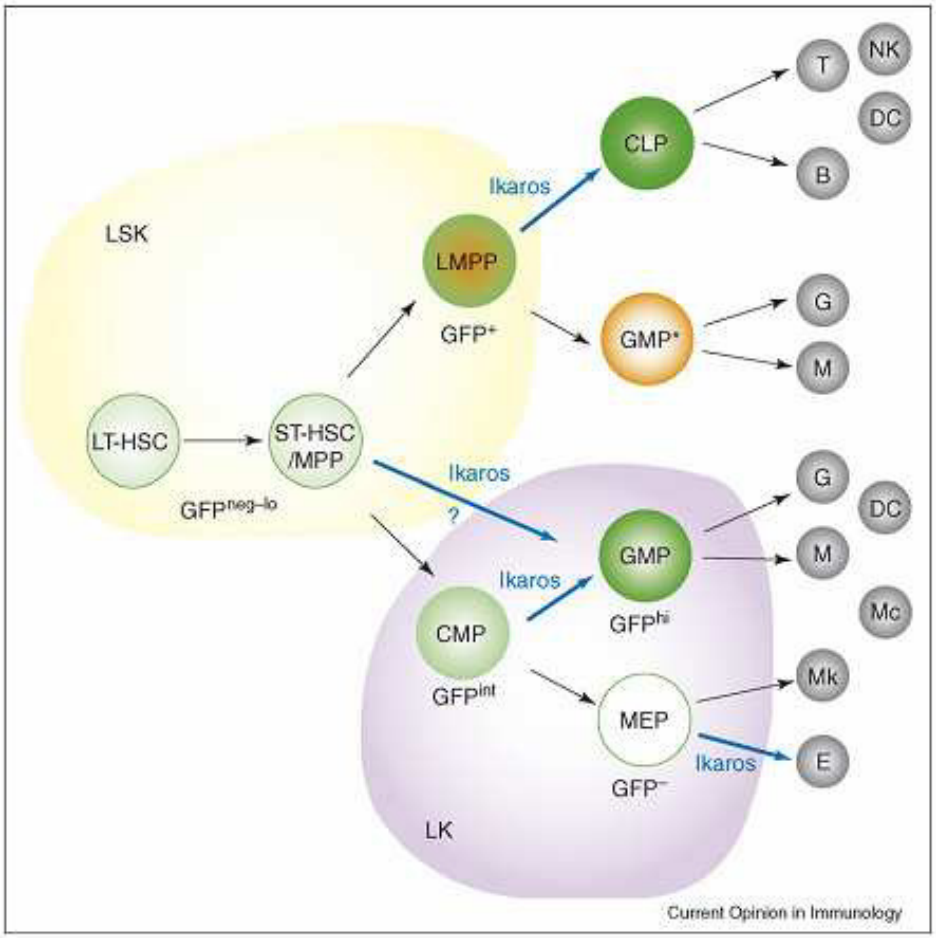


Figure 8. Role of Ikaros during hematopoiesis

Activity of the Ikaros–GFP reporter-gene expression cassette shows Ikaros expression level in the different hematopoietic populations. Ikaros is important for the development of both lymphoid and myeloid lineages starting in the LMPP and CMPP. In the absence of Ikaros, an LMPP-mediated pathway of granulocyte macrophage progenitor development predominates. In addition, Ikaros is important for specification of the GMP at the expense of MEP in the CMP and for the development of erythrocyte progenitors at the expense of megakaryocyte progenitors from the MEP. The star indicates an alternative developmental pathway for GMPs.

The role of Ikaros during hematopoiesis is presented in figure 8. The absence of B and NK cells and their precursors in mouse models, such as ($IK^{DN/DN}$) (Georgopoulos et al., 1994), ($IK^{-/-}$) (Wang et al., 1996), IK-Plstc $^{-/-}$ (Papathanasiou et al., 2003) suggests a role of Ikaros during the early stage of the hematopoiesis.

The T-cells are missing in IK-Plstc $^{-/-}$ and ($IK^{DN/DN}$) and present in ($IK^{-/-}$) mouse (Winandy et al., 1999), but show defects in the TCR checkpoints, bias of the CD4 to CD8 differentiation and fewer

γδT cells (Harker et al., 2002; Wang et al., 1996). The role of Ikaros is not limited to lymphoid differentiation only, because it regulates other functions in these cells, such as control of isotype selection during class switch recombination in B cells (Sellars et al., 2009) and regulation of the activation threshold of T and B cells upon antigen stimulation (Avithal et al., 1999; Kirstetter et al., 2002; Dumortier et al., 2006).

The myeloid lineage seems to be relatively unaffected by Ikaros deficiency in the different mutant mouse models. The myeloid $CD11b^{+}$ precursor population shows down-regulated expression of Gr1 marker, suggesting impaired myeloid differentiation, but without abnormalities in the neutrophil development and function (Wang et al., 1996; Dumortier et al., 2003).

Ikaros also plays an important role during the erythropoiesis as we can conclude from the abberant erythroid development and impaired hemoglobin switching in $IK^{-/-}$ mouse (Nichogiannopoulou et al., 1999; Bottardi et al., 2009).

I. 9. Ikaros expression in non-hematopoietic cells

The expression pattern of Ikaros is typical but not unique for the hematopoietic cells. Ikaros expression has been reported in hormone-producing pituitary cells, where it regulates the expression of the proopiomelanocortin promoter. Ikaros deficiency in vivo results in reduced circulating adrenocorticotrophic hormone levels, reduced body weight and diminished survival, as this phenotype is rescued by systemic glucocorticoid-hormone administration (Ezzat et al., 2005a). Ikaros is expressed in the adult hypotalamic neurons, in which Ikaros mediates a decrease in the acetylation of histone H3 of the proximal growth hormone (GH) promoter, leading to a decrease in its expression (Ezzat et al., 2005b). Expression of Ikaros and dominant negative non-DNA-binding isoforms was also detected in about 50 % of primary human pituitary adenomas (Ezzat et al., 2003). The expression of a human pituitary tumor derived-fibroblast growth factor receptor-4 (FGFR4) has been detected in pituitary tumors but not in the normal pituitary cells. The effects of Ik1 on wild-type FGFR4 promoter activity is histone de-acetylation, and transcriptional silencing of the gene, while Ik6 expression resulted in the opposite phenotype, suggesting a tumor suppressive role of Ikaros in human pituitary tumors (Ezzat et al., 2003).

I. 10. Ikaros family members

Ikaros family of transcription factors contain five members - Ikaros, Helios, Aiolos, Eos and Pegasus

I.10.1 Aiolos

The significant differences between the phenotypes resulting from a dominant negative and a null mutation of Ikaros led to the characterization of Aiolos as a novel member of the Ikaros family (Morgan et al., 1997). The Aiolos gene encodes a protein homologous to Ikaros that possesses the same DNA binding specificity as Ikaros. It is able to homo- and hetero-dimerize with Ikaros and other members of the Ikaros family. Aiolos is initially expressed in lymphoid committed progenitors and is strongly up-regulated during the differentiation of these cells into pre T and pre B cell precursors. Human CD34+ progenitors do not express Aiolos; B cells express higher levels than NK and T cells, while the expression level of Aiolos in monocytes is almost undetectable. The most obvious difference of Aiolos expression at the subpopulation level is the difference between CD56 Bright and CD56 Dim NK cells (Billot et al., 2009). In addition to hematopoietic cells, its expression has been also detected in non-hematopoietic cell lines such as MCF-7, SW480, HEK, PC3 and HeLa (Billot et al., 2010). Similarly to Ikaros, Aiolos is able to both activate and repress transcription, as its activating properties are stronger than these of Ikaros (Morgan et al., 1997; Koipally et al., 1999). The Aiolos-null mutation results in hyper-proliferative peripheral B cells upon BCR-mediated in vitro stimulation. Even in the absence of immunization, an increased number of T cell–dependent B cell responses and elevated serum levels of IgG, IgE and auto-antibodies are detected. The lack of marginal zone and peritoneal B cells, as well as development of B cell lymphomas are also typical features for these mice (Wang et al., 1998). Aiolos is also implicated in the maintenance of long-term immunity because it is required for the generation of long-lived, high affinity bone marrow plasma cells – the main producers of high affinity antibodies during the secondary immune response (Cortés and Georgopoulos, 2004).

Similarly to Ikaros, Aiolos is implicated in the regulation of anti-apoptotic protein metabolism by regulating Bcl-2 expression and its cellular localization through direct binding and activation of Bcl-2 gene promoter (Romero et al., 1999). Aiolos is also able to interact with another anti-

apoptotic protein – Bcl-xL in IL-4-stimulated cells. IL-4 stimulation does not promote nuclear translocation of Aiolos or Bcl-xL, but induces tyrosine phosphorylation of Aiolos required for dissociation of Bcl-xL, thus promoting its anti-apoptotic functions (Rebollo et al., 2001). Despite its significance, the tyrosine phosphorylation of Aiolos in primary human lymphocytes is not implicated in the malignant transformation leading to leukemia (Antica et al., 2007).

In addition, experiments performed on the chicken B-cell line DT40 suggest a role of Aiolos in the B-cell receptor (BCR) signaling induced pre-mature B-cell apoptosis, through elevation of cytochrome C release from the mitochondria (Kikuchi et al., 2009).

I.10.1.1 Aiolos in leukemogenesis

Several reports implicate Aiolos in malignancies such as leukemia. Initially Aiolos expression has been identified to be differentially regulated at the epigenetic level in human tumoral cell lines and primary cells (Duhamel et al., 2008a). Aiolos overexpression due to euchromatin epigenetic modification of its promoter can be observed in B-cell chronic lymphocytic leukemia (B-CLL) (Billot et al., 2011). Promotor DNA demethylation and enrichment of activation associated histone markers, such as the dimethylation of the lysine 4 on histone H3 result in constitutive access of its upstream activator nuclear factor – κB and Aiolos upregulation in CLL. The increased Aiolos levels influence the survival of leukemia cells by upregulating the expression levels of Bcl-2 family members. Interestingly, the increased Aiolos protein levels in leukemic cells from B-CLL patients are not always due to epigenetic changes of its promoter elements, but for reasons that have yet to be identified (Duhamel et al., 2008b).

Similarly to Ikaros, Aiolos is also presented by different splicing isoforms, suggesting its complex role in the regulation of B lymphocyte function. Surprisingly, the leukemic state is not necessarily linked with deregulated expression of its splicing variant. Both normal and leukemic B lymphocytes express multiple isoforms of the human Aiolos gene (Liippo et al., 2001), as more than 80% of expressed Aiolos is of the full length Aio1 isoform. In addition, a homogeneous overexpression of the total amounts of Aiolos isoforms is observed in the B cells of CLL patients (Duhamel et al., 2008b). Interestingly, Aiolos and its isoforms have been reported to be

deregulated in the primary immunodeficiency disease - Common variable immunodeficiency (CVID) (Billot et al., 2009). The study reveals three new Aiolos isoforms in CVID patients, which are non-detectable in healthy donors, as well as protein up-regulation and different cellular distribution of Aiolos in T and B cells from CVID patients compared to healthy donors.

I.10.2 Helios

Helios was identified as the third Ikaros family protein in 1998 as a part of Ikaros complexes purified from an immature T cell line. Alignment of the primary sequences of Helios, Ikaros and Aiolos shows that the DNA-binding, transcriptional activation and dimerization domains are functionally conserved. Similarly to Ikaros, Helios can function as a transcriptional activator and encodes two protein isoforms that are able to recognize similar DNA sequences and to associate with various Ikaros isoforms. Expression of Helios was detected in the embryo, in adult hematopoietic stem cells and was further restricted to a subset of cells in the T cell lineage (Kelley et al., 1998; Hahm et al., 1998). The information about Helios function comes from studies that use gain of function mouse models in order to explore the role of this protein in B-lymphocyte development. The use of transgenic mice that express Helios under the control of an Ig μ enhancer shows an unaltered commitment to the B cell lineage and normal numbers of bone marrow and splenic B cells. However, these B cells exhibited prolonged survival, enhanced proliferation, hyper-responsiveness to antigen stimulation and metastatic lymphoma suggesting that silencing of Helios is critical for normal B cell function (Dovat et al., 2005). In another study, full-length Helios or a Helios isoform that lacked the DNA-binding domain were overexpressed in hematopoietic progenitor cells of reconstituted mice. Constitutive expression of full-length Helios inhibited T-cell development at the double-negative stage within the thymus. Expression of the DNA-binding mutant doesn't lead to developmental abnormalities at early times after transplantation. However, 60% of animals that expressed this mutant developed an aggressive and transplantable T-cell lymphoma 4 to 10 months after transplantation, suggesting an important function for Helios during T-cell development (Zhang et al., 2007). Interestingly, inactivation of the Helios gene by homologous recombination does not impair the differentiation and effector functions of αβ and γδ T cells, NKT cells, and regulatory T cells, suggesting non-

essential role of Helios in T cell-development or functions that can be compensated by other members of the Ikaros family (Cai et al., 2009). Helios has also been reported to have a specific regulatory role in CD4 T cells during Th2 responses because of it is involved in the differentiation of CD4 T cells in T helper 2 and follicular helper T cells in vivo (Serre et al., 2011). An important finding reported by several groups is the upregulation of Helios levels in natural regulatory T cells (Tregs). Helios is upregulated in CD4+CD25+ regulatory T cells and chromatin immunoprecipitation (ChIP) experiments show that it binds to the FoxP3 promoter. siRNA – mediated knock-down of Helios down-regulates FoxP3 levels, resulting in significantly attenuated T_{reg} suppressive function (Getnet et al., 2010). An interesting finding is the observation that expression of Helios can be used to differentiate thymic-derived from peripherally induced foxp3+ T regulatory cells (Thornton et al., 2010). This study demonstrates that Helios is expressed in all CD4+CD8- Foxp3+ thymocytes, but not in naive T cells induced to express Foxp3 in vitro by TCR stimulation in the presence of TGF-b. This finding defines Helios as a potentially specific marker of thymic-derived Treg cells.

I.10.2.1 Helios in leukemogenesis

The high degree of homology of the functional domains of Helios and Ikaros suggests that Helios also might be implicated in malignancies, such as leukemia. In fact, the expression of aberrant Helios isoforms has been reported in T-cell acute lymphoblastic leukemia (Nakase et al., 2002). This study reports an overexpression of novel short isoforms of Helios (Hel-5, Hel-6, Hel-7 and Hel-8) in T-lymphoid cell line (HD-Mar) derived from patient with Hodgkin's lymphoma. The isoform Hel-5 which lacks the repressor function can associates with the full-length isoforms of the Ikaros gene family, Ikaros, Aiolos and Helios, and inhibits their DNA binding activity when present in excess, exerting a dominant-negative effects on the full-length isoforms of the Ikaros gene family (Tabayashi et al., 2007).

I.10.3 Eos and Pegasus

The expression of the other Ikaros family members Eos and Pegasus is not restricted to the hematopoietic system. Eos has been detected in the developing nervous system (Honma et al., 1999), as well in the skeletal muscle cells (Yeung et al., 2012). Similarly to Ikaros, Eos is able to associate with C-terminal-binding protein 1 (CtBP1) and to establish repression of reporter gene expression (Pedromo et al., 2002). Eos has been shown to mediate Foxp3-dependent gene silencing in CD4+ regulatory T cells, an event maintaining their suppressive abilities (Pan et al., 2009). This study shows that Eos directly interacts and forms a complex with Foxp3, which recruits the CtBP1 transcriptional repressor. The complex is further responsible for the establishment of epigenetic silencing marks and gene silencing. siRNA-mediated Eos knockdown abolishes T_{reg} mediated suppression and results in the appearance of unusual effector T-cell phenotype for this population. Eos has also been found to participate in the osteoclast commitment from myeloid progenitors after an appropriate set of extracellular signals (Hu et al., 2007). In this case, Eos recruits the co-repressors CtBP-1 and Sin3A by means of two other transcription factors -MITF (microphthalmia-associated transcription factor) and PU.1 in order to initiate osteoclast differentiation. Eos also plays an important role during the erythroid development by down-regulating the transcription of the γ-globin gene (Yu et al., 2011). Interestingly, Eos can also act as a transcriptional activator in nervous cells. It forms a complex with the intracellular domain (ICD) of Neuregulin-1 in order to stimulate the transcription of postsynaptic density protein (PSD-95) promoter (Bao et al., 2004). Eos also has been recently found to be implicated in the regulation of myofiber gene program by activating the transcription of intronic miRNA - miR-499. Upon its interaction with transcription factor MyoD Eos participates in the formation of an active transcriptional complex which regulates the expression of the endogenous Myh7b/miR-499 gene in muscle cells (Yeung et al., 2012).

Pegasus is the last discovered member of Ikaros family, which is able to dimerize with all the members of the family through its C-terminal zinc fingers. Pegasus doesn't recognize the Ikaros binding sequence TGGGAA and represses transcription of the reporter genes only if their

promotors contain the GXXXGXXG motif. Based on in vitro experiments, Pegasus is assumed to work also as a transcriptional repressor (Pedromo et al., 2002).

I.11 Ikaros family in human hematopoietic malignancies

	IKAROS	HELIOS	AIOLOS
ALL - acute lymphoblastic leukemia	Single nucleotide polymorphism (a,b); Deletion (c);Point mutations or frameshift (e); Expression of Dominant Negative Isoform (f);	Deletion (c); Expression of Dominant Negative Isoform (I,j,k);	Deletion (c); Downregulation (l)
Non-Hodgkin follicular center cell lymphoma (FCC)	-	-	Downregulation (m)
AML - acute myeloid leukemia	-	-	Downregulation (l)
BCR-ABL1–positive adult ALL	Deletion (c,p); Expression of Dominant Negative Isoform (o);	-	-
CLL - chronic lymphocytic leukemia	-	-	Upregulation (d,g,h,l)
CML - chronic myelogenous leukemia	Deletion (c);	-	-
Myelomonocytic and monocytic leukemias	Expression of Dominant Negative Isoform (n);	-	-

Table 1 shows the implication of Ikaros family members in human hematopoietic malignancies. Different types of genetic lesions in different leukemia types can be seen for three of the Ikaros family members.

Table 1. Implication of Ikaros family members in different human hematopoietic malignancies.

Refferences: a. (Trevino et al., 2009); b. (Papaemmanuil et al., 2009); c. (Mullighan, 2009a); d. (Nuckel et al., 2009); e. (Mulligan, 2009b); f. (Nakase et al., 2000); g. (Billot et al., 2011); h. (Duhamel et al., 2008); i. (Tabayashi et al., 2007); j. (Zhang et al., 2007); k. (Nakase et al., 2002); l. (Matulic et al., 2009); m. (Antica et al., 2008); n. (Yagi et al., 2002); o. (Iacobucci et al., 2008); p. (Mulligan et al., 2008)

I. 12 Thymocyte development

T-cell development in mammals is a stepwise process sensitive to environmental influence. Thymocytes originate from pluripotent precursors in the bone marrow or fetal liver, which migrate to the thymus and differentiate into mature T cells. A few T-cell precursors migrate into the thymus per day, respond to this new environment by extensive proliferation. They initiate a T-cell differentiation transcriptional program, gradually turning off genes that allow differentiation to non T-cell lineages (Hayday et al., 2007; Petrie, H.T, 2003; Rothenberg, E.V., 2000). Once they undergo T-cell receptor (TCR) gene rearrangements and assemble functional TCR complexes, they mature into different T-cell lineages, including γδ T cells and αβ T cells. The αβ T cells further diverge into different sub-lineages, such as CD4+ T cells, CD8+ T cells, regulatory T cells (T-Reg cells) and natural killer T (NKT) cells, which have different functions. All these diverse lineages share an early developmental history because many of the key genes that are required in later T-cell functions are first expressed before TCR rearrangement (Rothenberg, E.V., 2008). The precursors of the T-cell lineage called lymphoid primed multipotent progenitors (LMPPs) can give rise to macrophages, dendritic cells (DCs), NK cells, B cells and T cells, but not erythrocytes or megakaryocytes (Adolfsson et al., 2005). The early stages of the T-cell development are TCR independent and occur during the migration of the immature pro-T-cells through different thymic environments (Figure 9)(Petrie, H.T, 2007).

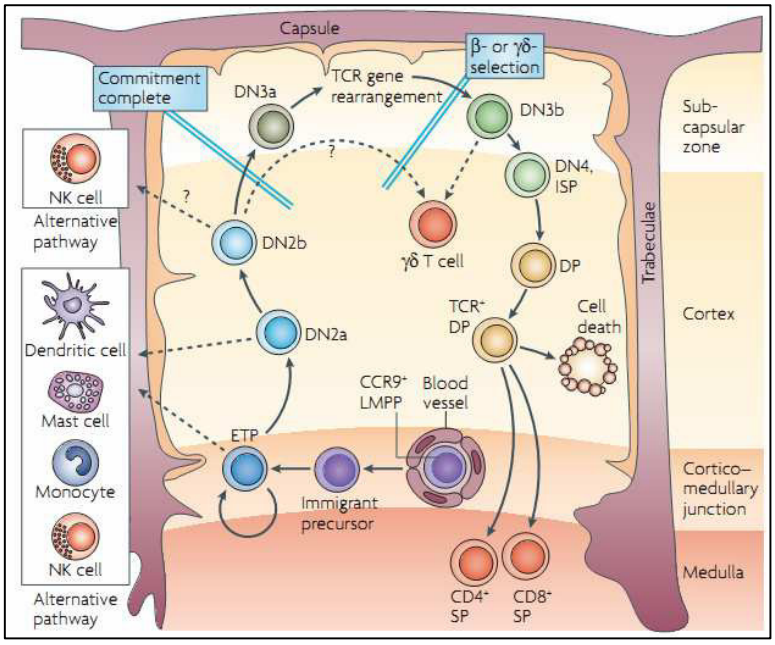
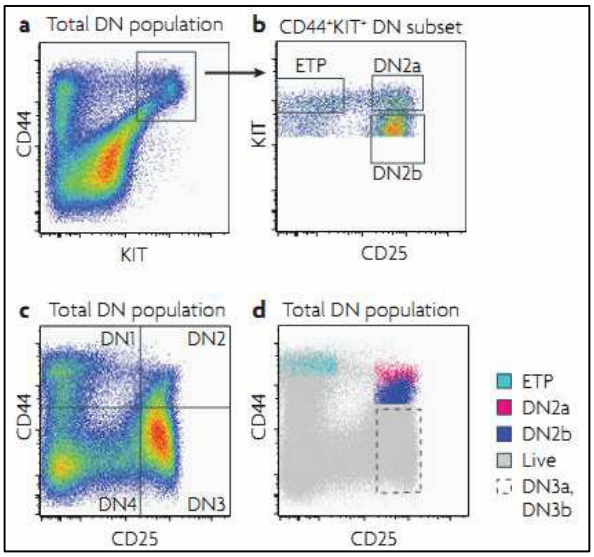


Figure 9. Early stages in T-cell development.

The ETP migrate, and differentiate from double negative (DN) to double positive (DP) to single positive (SP) stages, through the distinct microenvironments of the thymus. ETPs, DN2, DN3 and DN4 T-cell subsets can be distinguished by the differential expression of cell surface markers, such as CD44 and CD25. Dashed arrows show alternative developmental pathways that are still possible for ETPs and DN cells before they complete their commitment to the T-cell lineage. ETPs (early T-cell progenitors); CCR9 (CC-chemokine receptor 9); ISP (immature single positive); LMPP (ymphoid primed

multipotent progenitor); NK (natural killer); TCR (T-cell receptor). Adapted from (Rothenberg, E.V., 2008).

The thymus contains different zones in which T-cells at different development stages can be found. These stages can be distinguished by the expression of surface molecules such as CD44, CD25, CD4 and CD8 (Murphy et al., 2008). The early T-cell progenitors (ETPs) don't express the co-receptor molecules CD4 or CD8, but can be divided into six subsets – DN1, DN2a,DN2b, DN3a, DN3b and DN4 double negative. Initially, the ETPs (DN1) express c-kit, (receptor protein tyrosine kinase, which binds the growth factor - stem cell factor (SCF)), CD44 but not CD25 (Figure 10) (Rothenberg, E.V., 2008).



from (Rothenberg, E.V., 2008).

Figure 10. Phenotypic markers for the transitions through specification and commitment.

(A) Flow cytometry profile of expression of CD44 and KIT by CD4–CD8– double negative (DN) thymocytes from adult mice. (**B**) Profile of expression of KIT and CD25 by CD44+KIT+DN thymocytes, distinguishing early T-cell precursors (ETP), DN2a and DN2b subsets. (**C**) Flow cytometry profile of expression of CD44 and CD25 by CD4–CD8– double negative (DN) thymocytes. Conventional DN1, DN2, DN3 and DN4 quadrants are marked. (**D**) Location of ETP, DN2a, DN2b and DN3 cells within the CD44 and CD25 expression distribution of the whole DN population. Adapted

Interestingly, the ETPs can choose between maintaining their ability to proliferate without further differentiation and their T-cell commitment towards the DN2 stage. During the DN1-DN2 transition, the thymocytes begin to express CD25 (the alpha chain of the IL-2 receptor) on their surface. These cells actively proliferate while acquiring their first T-cell features. The T cells that have reached the DN3 stage stop their proliferation and increase TCRβ gene rearrangement, initially Dβ to Jβ and subsequently Vβ to DJβ. Those that fail to produce functional β-chain cannot survive this stage and are eliminated by apoptosis. DN3 T cells that succeed in making in-frame gene rearrangements will become activated by TCR-dependent selection (these are referred to as DN3b cells) (Taghon et al., 2006). The productive assembly of the CD3:preTCR complex, containing the TCR β-chain, CD3 and the surrogate pre-TCR α chain is performed during the DN4 stage and results in cell proliferation, blocking further β-chain gene rearrangement (allelic

exclusion), turning on the expression of CD4 and CD8 to become double positive (DP) cells. The main event during the DP stage is the rearrangement and expression of TCR α chain, which replaces the pTα in order to pair the β-chain, producing a functional α β-TCR (Figure 11).

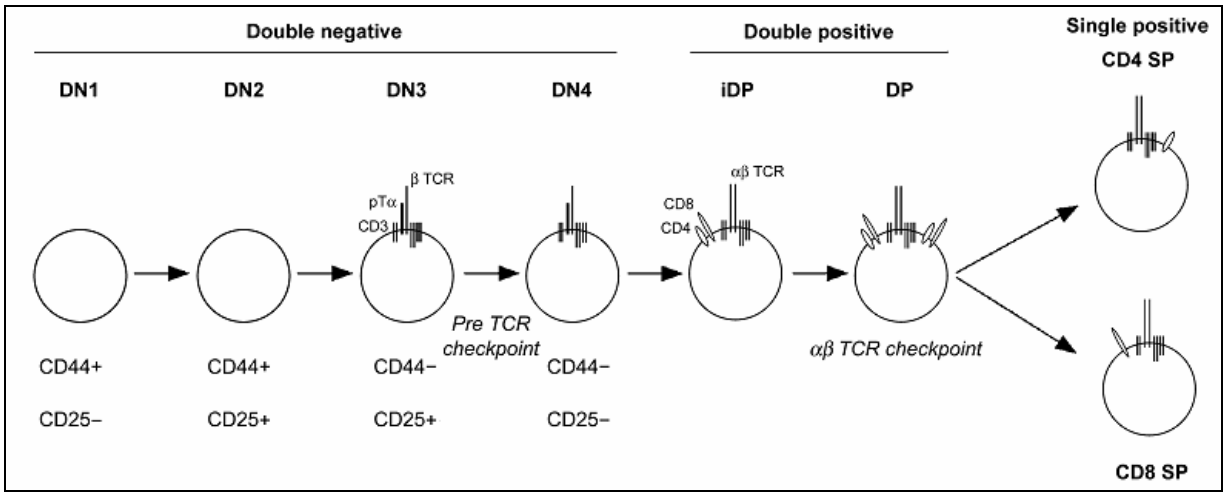


Figure 11. Schematically represented murine T cell development in the thymus.

The early stages of T cell development in the thymus are distinguished by CD44 and CD25 expression on double negative (DN) cells. Selection of a functional pre-T cell receptor (TCR) is followed by the upregulation of the coreceptors CD4 and CD8 to become double-positive (DP) cells. Following αβ TCR selection, DP cells downregulate CD4 or CD8 to become CD8 SP or CD4 SP cells, respectively, prior to export into the periphery. Adapted from (Miosge and Goodnow, 2005)

This prepares them for positive and negative selection to generate mature CD4+ or CD8+ αβTCR T cells, according to their avidity for the self-peptide:MHC (major histocompatibility) complex (Starr et al., 2003). The DP cells that express TCRs with low avidity to self-peptide-MHC complex are not able to get a positive survival signal and therefore die by neglect. Those that pass this first round of positive selection are subjected to a negative selection, which eliminates by apoptosis the cells with very high avidity to self-peptides. This is an important step during the T-cell development, leading to the removal of the autoimmune thymocytes (Blackman et al., 1990). Thus, about 98 % of the DP cells are eliminated in these two rounds of selection, allowing only about 2 % of DP cells expressing TCRs with medium avidity for self-peptides to survive and differentiate in single positive SP cells.

The decision between CD4 and CD8 lineage commitment happens between the DP-SP transition period, depending on the type of the MHC used during the positive selection. Cells selected on

MHC class II molecules, give rise to CD4+ SP cells, while those selected on MHC class I will develop in CD8+ SP cells (Sha et al., 1990).

The development of γδT cells is similar to the one of αβTCR cells (Figure 12). The rearrangement of the β, γ and δ chains is almost simultaneous and occurs during the DN2-DN3 stages. The cells that fail to pass both β and γδ-selection die by apoptosis. The decision of the DN cells to express γδ or αβ TCR has been reported to be dependent on the strength of the signal that the newly-formed TCR complex delivers (Hayes and Love, 2006). The engagement of γδTCR results in stronger signal than pre TCR formed by pTα and the TCR β-chain. Thus , in the first case the commitment will be in γδT direction, while in the second, αβT cell will be generated.

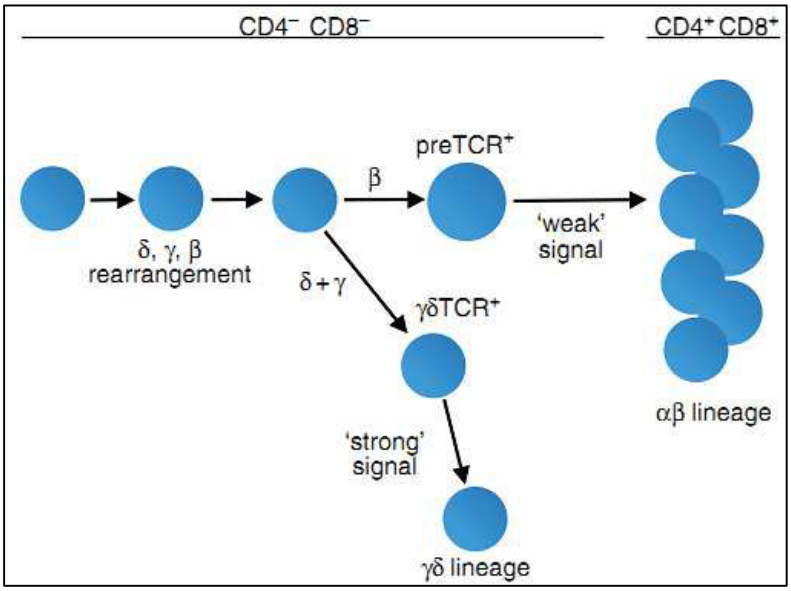


Fig. 12. Signal strength model for αβ/γδ lineage choice.

Quantitative differences in T-cell receptor (TCR) signal strength specify cell fate, such that an immature double negative (DN) thymocyte expressing the preTCR receives a "weak" signal and chooses the αβ lineage, whereas one expressing the γδ TCR receives a "strong" signal and chooses the γδ lineage. Adapted from (Hayes and Love, 2006).

I. 13 The role of thymic epithelium in thymocyte development

Thymocyte development is dependent on the combination of receptor ligands and growth factors provided by the thymic epithelium, which trigger and support pro-T-cell differentiation, proliferation and survival (Jenkinson et al., 2006; Boehm et al., 2006; Schmitt et al., 2005). The most critical for the thymocytes is the stimulation performed by ligands for the Notch cell-surface receptors - delta-like ligand 1 (DLL1) and DLL4 (Maillard et al., 2005). This signalling pathway initiates and sustains the T-cell lineage program throughout the pro-T-cell stages. The presentation of these two ligands by stromal cells is indispensable for the successful culture of T-cells in vitro (Schmitt et al., 2002). The thymic epithelium also produces cytokines and ligands for other signalling pathways, such as the KIT ligand (SCF) and interleukin-7 (IL-7). The migration of the pro-T cell compartment through different thymic zones provides diverse set of microenvironmental signals that help to drive T-cell differentiation programming (Petrie et al., 2007). In addition to the described interactions the early T-cell development can be influenced by more mature thymic T cells (Anderson et al., 2006; Silva-Santos et al., 2005).

I. 14 Core-group transcription factors involved in T-cell development

T-cell development depends on a panel of transcription factors that are important during the different stages of the differentiation. One typical feature of the transition of the T-cell precursors from the ETP to the DN3 stage is the major change in the expression of transcription factors involved in the cell-lineage plasticity and those implicated in the regulation of TCR rearrangement. One of them is Notch and the signaling mediated by its intracellular domain (NIC). NIC-mediated signalling converts the transcription factor RBPj (recombination site binding protein j) from a repressor to an activator (Maillard., 2005; Tanigaki et al., 2007), an event which activates DN2 and DN3 stage specific genes, such as *Ptcra, Hes1* (hairy, enhancer of split 1) and *Il2ra* (Reizis et al., 2002; Deftos et al., 2000). Interestingly, Notch signalling alone is not sufficient to activate or maintain the T-cell development without a core group of diverse transcription factors that must

work with Notch during the T-cell development (Anderson et al., 2006; Staal et al., 2001). This core group of factors includes: GATA3 (Ho et al., 2007), MYB, RunX1–CBFβ complexes; basic helix-loop-helix E proteins such as E2A (Murre et al., 2005), HEB, TCF1, the zinc finger repressor GFI1 (growth factor independent 1 transcription repressor), Bcl11b (Li et al., 2010) and Ikaros family members (Rothenberg, E.V., 2008)

I.15 Brief description of the stage-specific transcription factors in T-cell development

Thymocyte differentiation also depends on factors that do not have sustained functions throughout the process of development. One such factor is Pu.1, which is vital during pre-thymic precursor development, but switched off after the DN2a - DN2b transition stage (Rothenberg, E.V., 2008). Not all transcription factors expressed in LMPPs are turned off during the T-cell differentiation. A number of transcription factors continue to be expressed stably from multipotent progenitor stages to the cell stages when β-selection occurs (Tydell et al., 2007). Interestingly, only a limited set of transcription factors are specifically turned on at the onset of T-cell lineage gene expression in the DN2 stage, including Bcl11b, HEBAlt (Tydell et al., 2007; Wang et al., 2006) and GLI2 (glioma-associated oncogene 2) a transcription factor involved in the hedgehog signalling pathway (El Andaloussi et al., 2006). The DN3 stage is marked by the expression of several additional factors such as ETS1 and RunX1–CBFβ, which work in collaboration (Goetz et al., 2000; Gu et al., 2000). In addition to ETS1, ETS2, TCF-1 (T-cell factor 1), LEF1 (lymphoid-enhancer-binding factor 1) are also included in a group of transcription-factor genes, which are strongly up-regulated at the DN2 to DN3 stage transition (David-Fung et al., 2006). The DN3 stage is also distinguished by high expression levels of the Pu.1-related factor SPIB and Hes1, which are turned off after β-selection.

I.16 Description of the known posttranslational modifications of transcription factor Ikaros

I.16.1 Phosphorylation

The first evidence that Ikaros functions are regulated by posttranslational modification comes from a study, which shows that Ikaros ability to negatively regulate the G1-S transition can be modulated by phosphorylation of a serine/threonine-rich conserved region in exon 8 (Gomez-del Arco et al., 2004). Ikaros phosphorylation at positions 63, 385, 387, 389, 393 and 394 is induced during the G1-S transition. The phosphorylation negatively modulates Ikaros's activity as a regulator of the G1-S transition in response to signaling events that decrease its DNA binding activity. Mutations that abolish Ikaros phosphorylation increase its ability to impede cell cycle progression and its affinity for DNA. Casein kinase II (CKII) is the major kinase involved in Ikaros phosphorylation, whose increased activity in lymphocytes has been reported to be an event leading to a cell transformation and leukemia (Dovat et al., 2011). Similarly, in another study, Ikaros phosphorylation has been found to regulate its recruitment to pericentromeric heterochromatin (PC-HC) (Gurel et al., 2008). This study identifies the phosphorylation of serine residues at positions 13 and 294 to be crucial for the DNA-binding of Ikaros. Introduction of phosphomimetic substitutions at these sites, mimicking constitutively phosphorylated serines, abolishes pericentromeric targeting and DNA-binding of Ikaros in electromobility shift assays (Gurel et al., 2008). The amino acid residues described to be phosphorylated in the mentioned above studies are localized outside the DNA-binding domain of Ikaros. Notably, their phosphorylation seems to be critical for the DNA-binding ability of Ikaros, outlining the importance of this posttranslational modification in its functions. As a part of the large Kruppel-like family of transcription factors, Ikaros and its family members share a significant homology of the linker sequences separating their zinc finger motifs. In fact, the linker separating finger motifs is highly conserved and can be defined as TGEKP in more than 5000 occurrences (Dovat et al., 2002). Interestingly, the three linkers in the DNA-binding domain of Ikaros are phosphorylated during mitosis, similarly to another zinc finger protein - Sp1. While the introduction of phospho-abolishing mutations has no effect, the phosphomimetic substitutions at these positions interfere with the DNA-binding and pericentromeric localization of Ikaros (Dovat et al., 2002). In agreement with these data, one more study reported that reduction in DNA-binding affinity of Cys2His2 zinc

finger proteins can be mediated by phosphorylation of their linker motifs (Jantz and Berg, 2004). In this fluorescence-based DNA-binding *in vitro* assay, the phosphorylation of a single linker reduced binding affinity 40-fold, whereas phosphorylation of both linkers reduced binding affinity 130-fold. These assays based on the use of purified components demonstrate that linker phosphorylation results in a significant reduction of the DNA-binding affinity. This result suggests a model in which, a single cell cycle-dependent Ser/Thr kinase could simultaneously inactivate a large number of zinc finger transcription factors (Jantz and Berg, 2004). Interestingly, Ikaros phosphorylation has been reported to regulate not only its pericentromeric localization but also its stability via ubiquitin/proteasome dependent pathway (Popescu et al., 2009). This study reports that Ikaros interacts with protein phosphatase 1 (PP1) via a conserved PP1 binding motif, RVXF, in its C-terminal end inside the first dimerization zinc finger. Point mutations of the RVXF motif abolish Ikaros-PP1 interaction resulting in decreased DNA binding, an inability to localize to PC-HC and rapid degradation of the protein. The introduction of phospho-abolishing (alanine) substitutions at CK2-phosphorylated residues increases the half-life of the PP1-non-binding Ikaros mutant, suggesting that dephosphorylation of these sites by PP1 stabilizes the Ikaros protein and prevents its degradation. In vivo experiments show that Ikaros is able to interact and to target PP1 to the nucleus, in which they both colocalize with PC-HC (Popescu et al., 2009). Their model suggests that Ikaros functions are controlled posttranslationally by the CK2 and PP1 pathways and that a balance between these two signal transduction pathways is essential for normal cellular function and for the prevention of malignant transformation. A convenient way to present the phosphorylated residues on Ikaros is to generate a map, on which all of them are mapped (Figure 13). The consensus sumoylation sites that have already been published are also shown.

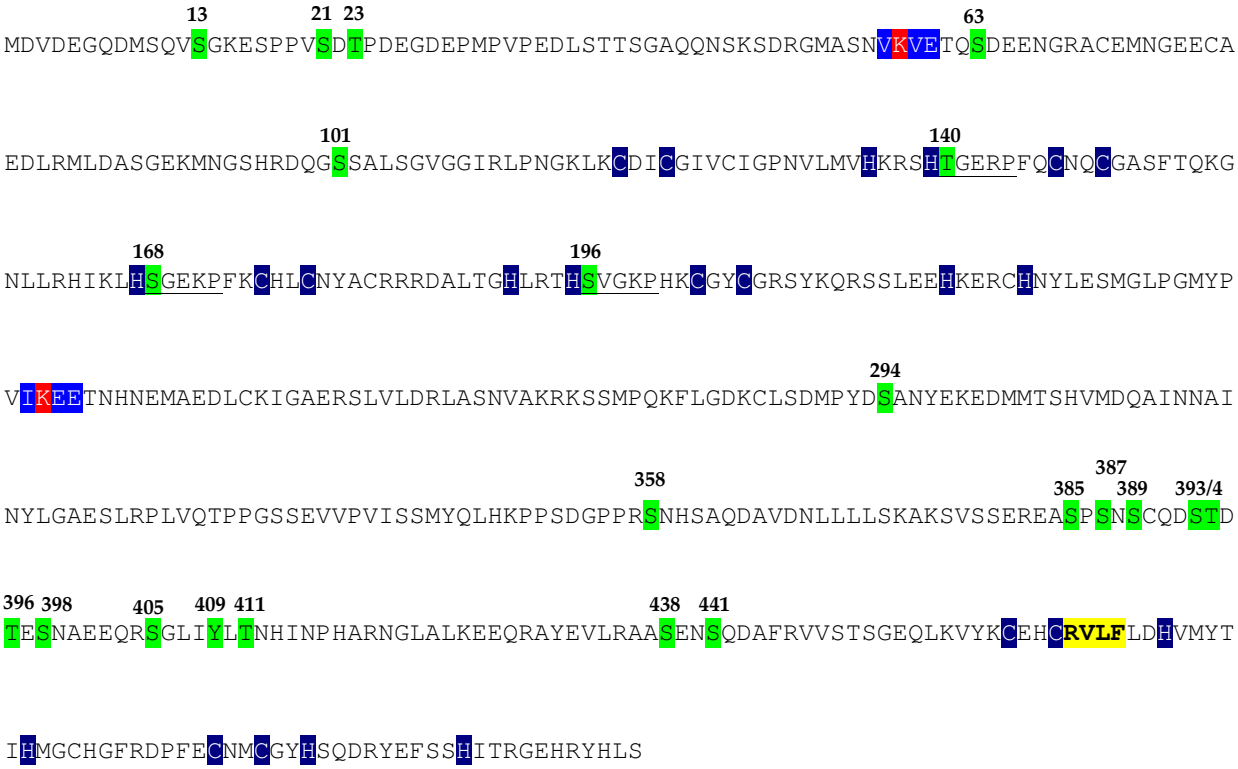


Figure 13. Ikaros map on which all the reported phosphorylated and sumoylated residues are depicted.

The consensus sumoylation sites are shown in blue, while the targeted lysine in the middle is shown in red. The Cys and His residues that shape each zinc finger are shown in dark-blue. The linker motifs between the N-terminal zinc fingers are underlined. The protein-phosphatase 1 (PP1) binding site in the first dimerization zinc finger is noted with highlighted in yellow bold shrift.

Figure 13 Ref: Concerning sumoylation: (Gómez-del Arco et al., 2005)

Concerning phosphorylation:

(Gómez-del Arco et al., 2004) – (S63, 385, 389, 394); (Gurel et al., 2008) – (S13, S101, 294, T23); (Sridharan R., Smale T., 2007) - (S63, 168, 358, 377, 385, 405, Y409, T394, 396, 411, 438, 441); (Popescu et al., 2009) - (S21, 389, 393, 398, T394, 396); (Dovat et al., 2002) – (S140, 168, 196);

I.16.2 Sumoylation

The small ubiquitin-like modifier or SUMO is a protein expressed in all eukaryotes (yeast, plants and animals), but is absent in bacteria and archaea. Mammals express four SUMO proteins: SUMO1, SUMO2, SUMO3 and SUMO4. A fourth member - SUMO4, is coded in the human genome, but it is not clear whether its product can conjugate to other proteins (Owerbach et al., 2005). Although SUMO1 and SUMO2/3 share the same basic conjugation machinery, their functions are different. For example, they conjugate to different target proteins, are differentially affected by isopeptidase enzymes, respond differently to stress, and can be distinguished by their ability (SUMO2/3) or inability (SUMO1) to form SUMO chains (Tatham et al., 2001).

SUMOylation is a posttranslational modification that can change the properties and regulate the function of a given protein. There are many types of protein modifications, such as the attachment of small groups, for example phosphate, acetyl or methyl groups, sugars or lipids. Alternatively, a protein can be modified through the covalent attachment of another, usually smaller, protein. The best known such modifier is ubiquitin, whose main function is to “tag” other proteins for degradation (Meulmeester and Melchior, 2008). There are a number of ubiquitin-like modifiers that have been identified, of which SUMO seems to affect the widest range of proteins (Table 1) (Welchman et al., 2005).

Table 2

Conjugatable human ubiquitons

Ubiquitin-like protein*	Sequence identity to ubiquitin (%)	Substrate(s)	Attributed function(s)
NEDD8 (Rub1)	58	Cullins, p53, MDM2	Regulation of E3s
SUMO-1 (Smt3)	18	Many	Nuclear localization, transcriptional regulation, antagonizing ubiquitylation
SUMO-2 and -3	16	C/EBPβ1, topoisomerase II	Transcriptional regulation, mitosis
ISG15/UCRP‡	29, 37	PLCγ1, JAK1, STAT1, ERK1/2, serpin 2a	Immune responses, interferon signal transduction
FAT10‡	29, 36	Unknown	Apoptosis, cytokine signal transduction
FUB1	37	TCR-α-like protein, Bcl-G	T-cell activation
UBL5 (Hub1)	22	Unknown	Pre-mRNA splicing
URM1	12	Ahp1	Oxidative stress response, nutrient sensing (through the TOR signalling pathway)
ATG8	10	Phosphatidylethanolamine	Autophagy, cytoplasm-to-vacuole targeting
ATG12	17	Atg5	Autophagy, cytoplasm-to-vacuole targeting

Although SUMO is ubiquitin-related protein with similar mechanism of conjugation and de-conjugation to other proteins, the functional consequences of sumoylation might be very different. The SUMO family has been shown to modulate many protein functions and processes, such as nuclear localization, transcriptional regulation, protein stability (indirectly by antagonizing or promoting ubiquitylation) (Gill G., 2004; Tatham et al., 2008; Lallemand-Breitenbach et al., 2008; Desterro et al., 1998). Many SUMO targets contain a consensus sumoylation motif of four amino acids that is recognized by the E2 enzyme Ubc9 (ΨKxE). In this motif the sumoylated lysine is preceded by a hydrophobic amino acid (Ψ) and followed by any aminoacid plus acidic residue - l/L/V-K-x-E/D. Many targets are modified at sites other than the consensus motif through various mechanisms or proteins that are not SUMO targets can contain this consensus motif. An example for non-consensus sumoylation is when the E2 conjugating enzyme recruitment is done by means of a SIM (SUMO interacting motif) in the target (Meulmeester and Melchior, 2008). In addition to canonical four amino acid SUMO consensus motifs, longer sequences that include both SUMO consensus motifs and additional elements have been identified in some SUMO substrates. These include phosphorylation-dependent SUMO motifs (PDSMs) and negatively charged amino acid-dependent SUMO motifs (NDSMs) (Figure 14) (Gareau and Lima, 2010).

Figure 14. SUMO consensus and SUMO interacting (SIM) motifs. Adapted from (Gareau and Lima, 2010)

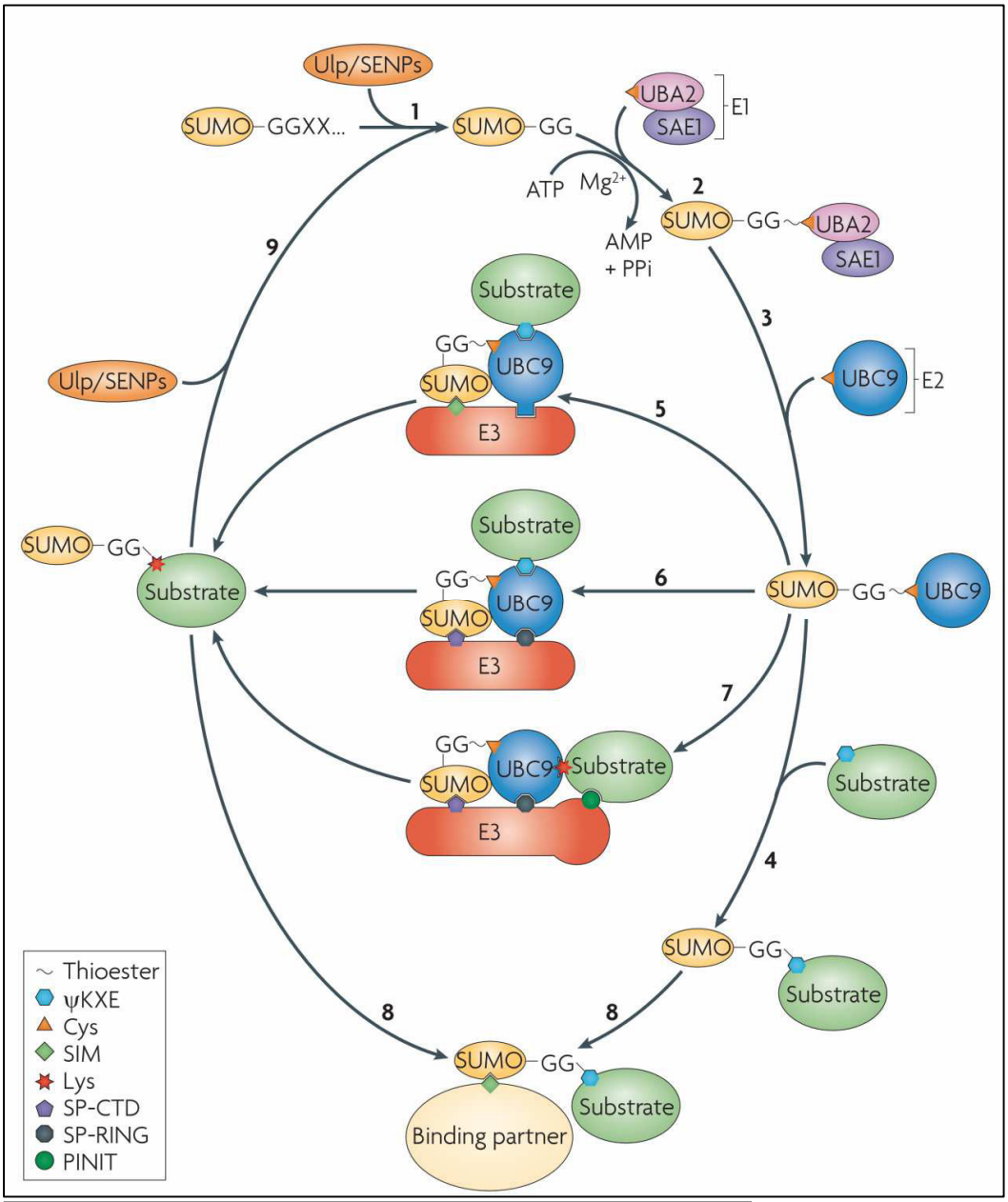
Different types of consensus sumoylation motifs (**A**) and SUMO interacting (SIM) motifs shown in blue (**B**)

PDSMs occurs in proteins that are substrates for modification by Pro-directed kinases (Yang et al., 2006) and comprise a SUMO consensus motif located adjacent to a phosphorylation site, ψKx(D/E)xxSP. In all known cases, phosphorylation increases levels of SUMO conjugation both in vitro and in vivo.

I.16.2.1 Mechanism of the sumoylation cascade

The SUMO conjugation cycle is presented in Figure 15. The SUMO protein is expressed as a precursor, which can be processed by ubiquitin-like protein-specific proteases (Ulps) and sentrin-specific proteases (SENPs) to its mature form (**STEP 1**). This cleavage reveals a carboxy-terminal di-glycine motif, which is then adenylated by the SUMO-activating enzyme subunit 1 (SAE1)–ubiquitin-like activating enzyme subunit 2 (UBA2) E1 complex in an ATP·Mg2+ -dependent manner and transferred to the catalytic Cys of the UBA2 subunit (**STEP 2**). Upon this activation step, SUMO is transferred to the catalytic cysteine of the E2 conjugating enzyme, ubiquitin-like conjugating enzyme 9 (UBC9) (**STEP 3**), which triggers its conjugation to a substrate in an E3 ligase-independent manner through recognition of SUMO consensus motifs (ΨKXE) that contain a Lys acceptor residue (**STEP 4**). In the case for Ran-binding protein 2 (RanBP2), the E3 ligase is able to maintain the E2-SUMO thioester in an appropriate conformation for catalysis without directly contacting the substrate (**STEP 5**) (Reverteret al., 2005). The Siz (in yeast) and PIAS (protein inhibitor of activate STAT) E3 ligases contact the E2 and SUMO through their SP-RING and Siz/PIAS carboxy-terminal domain (SP-CTD) domains, respectively (**STEP 6**). In (**STEPS 5 and 6**) the substrate specificity is achieved by its direct interaction with the E2 enzyme. The Siz/PIAS family proteins also contain a PINIT domain, which is able to contact the substrate, as is the case for the substrate proliferating cell nuclear antigen (PCNA) (Yunus et al., 2009; Reindle et al., 2006) (**STEP 7**). The ability of the E3s to directly interact with the substrates is considered to be important when conjugation to non-consensus lysine residues occurs. SUMO-modified substrates can contact SUMO-binding proteins by means of their SUMO-interacting motifs (SIMs) (**STEP 8**). The desumoylation process is performed by Ulp and SENP proteases, resulting in free SUMO monomers, that may be used for another round of conjugation (**STEP 9**) (Gareau and Lima, 2010).

A.



B.

	E1	E2	E3	SUMO–cleaving protease
S. c.	Aos1·Uba2	Ubc9	Siz1 Siz2 (Nfi1)	Ulp1 Ulp2 (Smt4)
H. s., *M. m.,* *R. n.*	Aos1·Uba2 (SAE1,Sua1·SAE2)	Ubc9	PIAS1 (GuBP) PIAS3 (KChaP) PIASxαβ (ARIP3,Miz1) PIASy RanBP2 (Nup358) Pc2	SENP1 SENP2 (Axam,SuPr-1,SMT3IP2) SENP3 (SMT3IP1) SENP6 (SUSP1)

Figure 15. Schematical representation of Small ubiquitin-related modifier (SUMO) conjugation cycle and its components. Adapted from (**A**) (Gareau and Lima, 2010) and (**B**) (Johnson E., 2004)

(**A**) Note that the SUMO conjugation might occur in the absence (STEP 4) or in the presence (STEPS 5, 6 and 7) of a SUMO E3-ligating enzyme. (**B**) Proteins belonging to a different classes of enzymes are involved in the sumoylation cascade.

I.17 The role of sumoylation on the transcriptional regulation

The sumoylation have diverse effects on the regulation of the gene expression. Sumoylation of Kruppel-like transcription factor ZBP-89 by conjugation of multiple SUMO isoforms occurs at two conserved synergy control motifs flanking the DNA binding domain. This modification inhibits the functional cooperation between ZBP-89 and heterologous activators such as the glucocorticoid receptor. Thus, sumoylation of ZBP-89 exerts a strong inhibitory effect on the diversity of its synergistic interaction partners (Chupreta et al., 2007).

Kruppel-like transcription factor 5 (KLF5) is a crucial regulator of energy metabolism. Under basal conditions, KLF5 sumoylation is associated with transcriptionally repressive regulatory complexes containing unliganded peroxisome proliferator–activated receptor-d (PPAR-d) and co-repressors and thus inhibited the expression of genes, such as Cpt1b, Ucp2 and Ucp3. Upon agonist stimulation of PPAR-d, KLF5 is desumoylated and associates with transcriptional activation complexes containing both the liganded PPAR-d and CREB binding protein (CBP) (Figure 16). This activation complex increased the expression of Cpt1b, Ucp2 and Ucp3. Thus, sumoylation seems to be a molecular switch affecting function of KLF5 and the transcriptional regulatory programs governing lipid metabolism (Oishi et al., 2008).

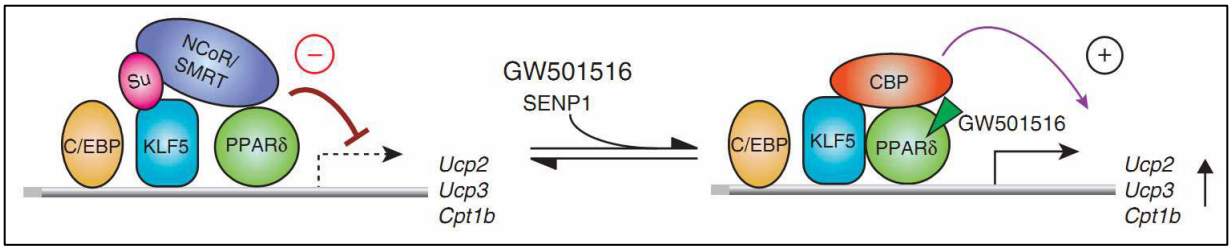


Figure 16. The model of the transcriptional regulatory programs governing expression of Cpt1b, Ucp3 and Ucp2 in skeletal muscle. Adapted from (Oishi et al., 2008).

Under basal conditions, SUMOylated KLF5 and unliganded PPAR-d interact with the corepressors NcoR and SMRT to form transcriptionally repressive complexes. The GW501516 ligand, however, initiates rapid local desumoylation that is followed by an exchange of co-regulators, chromatin remodeling and activation of transcription. In transcriptionally active complexes, unsumoylated KLF5 interacts with CBP and liganded PPAR-d.

The sumoylation of transcription factor Sp1 within the N-terminal negative regulatory domain of the protein has been reported to regulate its integrity. Unlike the sumo-deficient mutant, wild type Sp1 was determined to be N-terminally cleaved indicating that sumoylation and cleavage are coupled. Compared with Sp1, sumoylation-deficient Sp1 exhibited enhanced ubiquitin/proteasome-mediated cleavage and was a better transcriptional activator, while constitutively SUMO-1 modified Sp1 was deficient in proteolytic processing and repressed Sp1 transcriptional activity (Figure 17). Thus, sumoylation preserves the integrity of a negative regulatory domain thereby allowing for the inhibition of Sp1-dependent transcription (Spengler and Brattain, 2006).

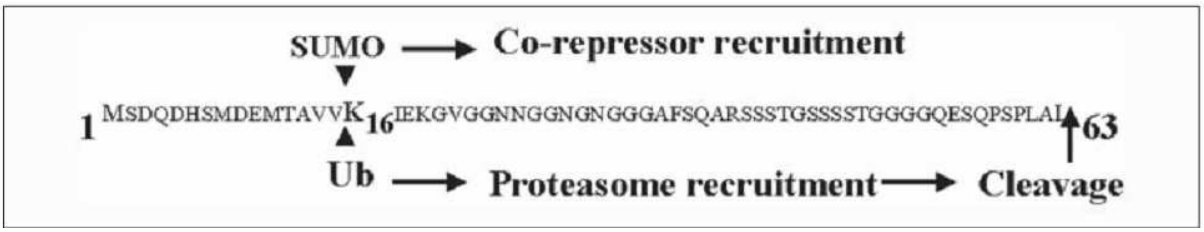


Figure 17. Posttranslational competition for lysine 16 governs Sp1 N-terminal cleavage. Adapted from (Spengler and Brattain, 2006).

The amino acids 1–63 of Sp1 are reported to be a primary recognition site for the proteasome-dependent cleavage at residue 63. Ubiquitin-conjugated lysine 16 assists in proteasome-mediated cleavage with the end result being a more active Sp1 transcription factor. SUMO-1-conjugated lysine 16 prevents cleavage and assists in recruitment of co-repressors.

Another transcription factor, whose functions are modulated by sumoylation, is the zinc finger protein Sp3. This GC box binding transcription factor is able to both activate and repress transcription. Endogenous Sp3 is sumoylated and localized to the nuclear periphery and in nuclear dots. SUMO-deficient Sp3 mutant acts as a strong transcriptional activator with a diffuse nuclear localization. Expression of SUMO-1 - Sp3 fusion protein represses Sp3-dependent transcription and relocalizes Sp3 to the nuclear periphery and nuclear dots. Thus, SUMO-1 modification is able to modulate the dual functional activity of this transcription factor (Ross et al., 2002; Sapetschnig et al., 2002; Spengler et al., 2005; Stielow et al., 2008).

Interestingly, the shorter Sp3 isoforms that result from internally initiated translation sites can also be modified by SUMO-1. The full length version of Sp3 is only a weak to moderate transcriptional activator of its target promotor of *SRC* gene, coding the non-receptor tyrosine

kinase pp60c-Src. Mutations preventing sumoylation of the shorter Sp3 isoforms are sufficient to convert them into potent transactivators. (Ellis et al., 2006).

CHAPTER 2

Materials and Methods

II. Plasmids and cloning procedures

II.1 Retroviral constructs

The retroviral MSCV plasmid Mig-R (IRES-GFP) was used for the cloning of all the deletion and point mutants. The generation of all the mutant constructs was performed by two-step fusion PCR using overlapping-end primers (for the deletion mutants) or overlapping primers, bearing a single point mutation K to R (for the point K to R mutants), using high-fidelity *Pfu* polymerase (Agilent) (CAT# 600153), according to the manufacturer's protocol. In case of the DMZF mutant, the amino acids L117/K118 and K459 were converted into M117/N118 and R459. The PCR was performed on Mig-R IK1-ER template, containing the coding sequence of Ikaros1 isoform fused to the ligand binding domain of the Estrogen Receptor (ER) with the following Mig-R primers:

Fw: 5' CAGGTCCCCTACATCGTGAC

Rev: 5' CGACATTCAACAGACCTTGC

The amplified and XhoI/EcoRI digested PCR products were purified by QIAGEN - QIAquick Gel Extraction Kit (CAT#28106) and cloned into XhoI/EcoRI digested Mig-R vector. The PMX-PIE (IRES-GFP) vector containing the puromycin resistance gene (Puromycin N-acetyltransferase - PAC) under the control of the hPGK promotor was used for the cloning of the FLAG-HA tagged full length wild type or ΔDOM Ikaros1 isoform by HindIII/EcoRI ligation.

The plasmid constructs used in the transient transfection assays were generated by cloning the IK1 point mutants into pTL-2 expression vector under the control of the viral SV40 early promotor. The IK1 point mutants were amplified from Mig-R-IK1-ER mutant templates with Fw (Kozac) and Rev (*stop codon*) Ikaros primers containing respectively XhoI and EcoRI restriction sites:

Fw: XhoI 5' AT**CTCGAG**CCACCATGGATGTCGATGAGGGTCA

Rev: EcoRI 5' AT**GAATTC***TTA*GCTCAGGTGGTAACGATG

II.2 Cell Lines

II.2.1 ILC87c

The T-cell line ILC87c originates from a mouse strain which expresses both floxed Ikaros gene (exon 7) and CRE recombinase under the control elements of Lck promotor, resulting in a T-cell specific deletion of Ikaros exon 7 and Ikaros null mutation. The cell line originates from IK-null thymus and shows no detectable expression of stable endogenous Ikaros.

II.2.2 149.3B

The T-cell line 149.3B originates from T-cell leukemia of a mouse strain with expression of constitutively active form of beta catenin (Kirstetter et al., 2006). In this mouse, the coding sequence of myc-tagged mutant beta catenin form (S33Y) is knocked in the ubiquitiously expressed R26 locus. This mutant beta catenin form cannot undergo phosphorylation and subsequent degradation, which makes this signaling pathway constitutively active and renders the T-cells hyper proliferative in the presence of endogenous Ikaros protein.

II. 3 Cell culture reagents

These T-cell leukemia cell lines were maintained in (RPMI1640 supplemented with w/25 HEPES; 10 % heat-inactivated fetal calf serum; 1 mM NaPyr; 1 % Pen/Strep).

The 293T human embryonic kidney cells were maintained in Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum; 1 % Pen/Strep).

Eco Phoenix – packaging cell line was maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g/l glucose; 10 % heat-inactivated fetal calf serum; 1 mM NaPyr; 1 % Pen/Strep).

All the reagents were purchased from Invitrogen.

II.4 4-OHT treatment

The cell lines expressing IK1-ER or its point or deletion mutants were treated with 100 nM 4-OHT diluted in Ethanol (SIGMA) for 24 hours prior lysis.

II.5 Transient transfections and viral production

For the transient transfections or virus production, respectively 293T cells or Eco Phoenix cells were transfected with mix of 5 μg of the indicated expression or retroviral plasmid DNA plus 5μg BSK carrier plasmid in final 125 mM $CaCl_2$.The transfection was performed with $CaPO_4$ method. Briefly, 9 x 10^6 cells were seeded 12 hours prior transfection in 100 mm plates to final 80 % of confluence. Transfection was carried out by mixing drop by drop the plasmid/$CaCl_2$ mix with 2 x transfection solution (pH 7.05, adjusted with 0.1 M NaOH) – containing 50 mM HEPES; 280 mM NaCl; NaH_2PO_4 1.5 mM in V/V ratio. After vigorous pipetting, the mixture is added to 6 ml DMEM medium containing 25 μM chloroquine (SIGMA) and gently pipetted onto the cells. 8 hours later, the transfection medium is removed and replaced by fresh medium and the cells are cultured for 24 hours.

In case of viral production, 24 hours post transfection the medium is replaced with 3.5 ml of fresh medium and the cells are cultured for additional 24 hours. 48 post transfection the supernatant containing the virus is filtered and used to infect the target cells.

II.6 Viral infection

The infections are carried out in 6 well plates seeded with 2 x 10^6 cells in 1.5 ml of medium. 0.5 ml of viral supernatant supplemented with 32 μg/ml polybrene is added to the target cells and the plates are centrifuged for 2 hours at 2.500 rpm. 24 hours later the medium is replaced by a fresh one and the cells are incubated for a new 24 hours. 48 hours post infection the infection efficiency was determined by flow cytometry analysis using Beckton Dickinson FACSCalibur.

II.7 RNA isolation

ILC87c cells expressing IK1-ER or its sumoylation deficient mutants were harvested and washed in ice cold PBS, then lysed in RLT buffer supplemented with beta-mercaptoethanol (10 µl/ml). Total RNA was isolated by QIAGEN RNeasy Mini or Micro kits (CAT#74106; CAT#74034) according to the manufacturer's protocol. The RNA purity and quantity was determined by Nanodrop 1000 (Thermofisher).

II.8 RT-PCR

RNA reverse transcription was performed with Superscript II reverse transcriptase (Invitrogen CAT#18064-014) according to the manufacturer's protocol. 350 ng or 1 µg of RNA was used in final 20 µl of reaction volume.

II.9 Microarray analysis

Total RNA was extracted from 24 hours Ethanol/4-OHT treated ILC87c cells expressing IK1-ER or its sumoylation deficient mutants using QIAGEN RNeasy Micro kit. RNA quality was verified by analysis on the 2100 Bioanalyzer (Agilent). All samples displayed a RNA Integrity Number greater than 8.

Biotinylated single strand cDNA targets were prepared, starting from 150 ng of total RNA, using the Ambion WT Expression Kit (Cat # 4411974) and the Affymetrix GeneChip® WT Terminal Labeling Kit (Cat # 900671), according to Affymetrix recommendations.

Following fragmentation and end-labeling, 1.9µg of cDNAs were hybridized for 16 hours at 45^{o}C on GeneChip® Mouse Gene 1.0 ST arrays (Affymetrix) interrogating 28,869 genes represented by approximately 27 probes spread across the full length of the gene. The chips were washed and stained in the GeneChip® Fluidics Station 450 (Affymetrix) and scanned with the GeneChip® Scanner 3000 7G (Affymetrix). Finally, raw data (.CEL Intensity files) were extracted from the scanned images using the Affymetrix GeneChip® Command Console (AGCC) version 3.2. CEL files

were further processed with Expression console version 1.1 using RMA algorithms using default settings.

Platform GEO: GPL6246. Reference manual:

The Ambion® WT Expression Kit Protocol P/N 4425209 Rev.B 05/2009

GeneChip® WT Terminal Labeling and Hybridization User Manual, for use with Ambion WT Expression Kit, Affymetrix, P/N 702808 Rev.1

II.10 Cell lysis

II.10.1 Total extracts

The cells were harvested, washed once in ice cold PBS and lysed in Total lysis buffer (1x PBS; 150 mM NaCl; 0.5 % NP-40, complete EDTA free protease inhibitor (Roche); Phosphatase inhibitor cocktail 3 (SIGMA); 10 mM NEM; 10 mM Iodoacetic acid).

II.10.2 Cytosolyc and Nuclear extracts

The cytosolyc extracts were prepared incubating a PBS washed cells in hypotonic buffer (HEPES 10 mM, pH 7.9; 1.5 mM $MgCl_2$; 10 mM KCl; complete EDTA free protease inhibitor (Roche); Phosphatase inhibitor cocktail 3 (SIGMA); 10 mM NEM; 10 mM Iodoacetic acid) for 30 min on ice, vortexing each 10 min. After spinning the extracts 5 min at 13 000 rpm (4^oC), the nuclear pellet was lysed in Total lysis buffer to get the nuclear fraction. The lysate is then vortexed and spun for 10 min at 13 000 rpm (4^oC) and the supernatant is used for western blot, Immunoprecipitation or kept at -80^oC.

II.11 Western Blot

Protein extracts equal to the indicated amounts were mixed with 5x sample buffer to final 50 µl and loaded on PAGE (6, 8 or 10 %), gradient (3 – 8 %) PAGE gels (Biorad Criterion) or gradient (4 – 20 %) NuPAGE (Invitrogen). The proteins were transferred on PVDF membrane (Immobilon), saturated in 5 % non-fat milk for 1 hour at room temperature (RT) and probed with the indicated antibodies.

II.12 Immunoprecipitation

500 µl of protein extracts equal to 50 million cells are pre-cleared with 20 µl of 50 % slurry protein A or protein G sepharose beads (SIGMA) for 30 min at 4^{o}C with rotation. The beads are pelleted by centrifugation 1 min at 13 000 rpm (4^{o}C) and the extract were transferred into tubes containing 50 µl of 50 % slurry protein A or protein G sepharose beads incubated overnight (O/N) with 2 µg of the indicated antibody. After O/N incubation with rotation at 4^{o}C, the beads were spun (3 min, 2000 rpm, 4^{o}C) and washed 3 x with Total lysis buffer without any inhibitors. The beads were then boiled in 25 µl of 3x Laemmli sample buffer and loaded on gel.

II.13 Luciferase reporter assay

HeLa cells (0.8 x 10^5) were seeded in 1ml of medium onto 24-well plates. Cells were transfected 24 h later with 300ng of expression plasmids (as indicated) to final 1 µg DNA per condition using JetPEI transfection reagent according to the manufacturer recommendations. To maintain the amount of the transfected plasmid DNA equal in each condition, the cells transfected with only one type of expression vector also received the required amount of empty expression vector.

Luciferase activity was determined by resuspending cold PBS-washed cells in 200 µl of reporter lysis buffer: (Tris-Phosphate 25 mM pH 7.8; EDTA 2mM; DTT 1 mM; Glycerol 50%; Triton X-100 1%) and shaking the plate for 10 min. at room temperature. 25 µl of each extract were subjected to luciferase assays using 50 µl of luciferase reaction mix [Luciferase buffer (Tris-Phosphate 20 mM pH 7.8; EDTA 0.1 mM; DTT 33.3 mM; $MgSO_4$ 2.7 mM; $MgCl_2$ 1 mM) and Luciferin 235 mM; ATP 265 mM; Coenzyme A 135 mM]. The luciferase activity was measured using BERTHOLD LB960 luminometer.

Beta-galactosidase activity was determined by resuspending 25 µl of each extract in 50 µl of reaction buffer (Na_2HPO_4 60 mM; NaH_2PO_4 40 mM; KCl 10 mM; $MgCl_2$ 1 mM; beta-mercaptoethanol 33 %) and 20 µl of ONPG (Ortho-nitrophenyl-β-D-galactopyranoside) 1.1 mg/ml. The mix was incubated 30 min at 37^0C and the reaction was stopped by the addition of 50 µl Na_2CO_3 1M. The OD was measured by spectrophotometer at 415 nm.

The luciferase activity was normalized to the beta-galactosidase activity, used as an internal transfection control.

CHAPTER 3

Results

During my PhD I worked on two different projects, the aim of which was to dissect the function of Ikaros as transcriptional activator and/or repressor in a relevant model system like a T-cell line. The aim of the first project was to characterize the role of a conserved domain among all Ikaros family members, known to be responsible for Ikaros-mediated transcriptional activation.

III. FIRST TOPIC

III.1 Studying the role of the transcription activating domain of Ikaros in mouse T-cell lines.

The Ikaros family of proteins contains three different functional regions. In addition to their four N terminal DNA-binding zinc fingers (Molnar and Georgopoulos, 1994), all the members have a conserved bipartite activation domain followed by two C-terminal zinc fingers required for the interaction with self and other family members (Sun et al., 1996; Morgan et al., 1997). Through this domain, Ikaros, Aiolos and Helios are able to activate transcription of reporter genes (Molnar and Georgopoulos, 1994; Kelley et al., 1998). The experiments that described the function Ikaros activation domain were done in yeast or mammalian cells like 3T3 fibroblasts and 293T cells, cell types that do not naturally express endogenous Ikaros. We wanted to address the role of this functional part of Ikaros in a model system more similar to primary cells. Thus, using PCR and standard cloning techniques I cloned FLAG-HA tagged wild type Ikaros1 and Ikaros1 delta DOM mutant (ΔDOM) in the retroviral construct PMX-PIE. The delta DOM deletion represents a deletion of a 147 amino acid stretch that contains the activation domain of Ikaros. It includes also 3 conserved among the Ikaros family motifs and is longer than the activation domain itself (Figure 18). In the PMX-PIE construct the expression of the gene of interest is coupled with the expression of enhanced green fluorescent protein (eGFP) by internal ribosomal entry site (IRES). This allows monitoring the level of the expressed protein by flow cytometry (FACS) for the GFP expression. In addition, the retroviral construct contains a puromycin resistance gene (Puromycin N-acetyltransferase or PAC) under the control of the hPGK promotor, which allows the selection of the infected cells after puromycin treatment. For these experiments I used a T-cell line called

ILC87c as a model system for infection and overexpression of WT Ikaros or ΔDOM Ikaros mutant. It originates from a mouse strain which expresses both floxed Ikaros gene (exon 7) and CRE recombinase under the control elements of Lck promotor. It is a T-cell specific promotor that becomes active during the early stages of T-cell development (DN1 – DN2), deleting Ikaros exon 7 and resulting in an Ikaros null mutation. The ILC87c cell line originating from IK-null thymus, does not express stable endogenous Ikaros protein and it is therefore suitable as an Ikaros overexpression system. Thus, to characterize the functional significance of Ikaros activation domain in T-cell context, ILC87c cells were infected with either FLAG-HA tagged WT or ΔDOM Ikaros and the PMX-PIE (empty vector) as a negative control. Twenty four hours after infection the cells were selected with medium containing puromycin for six days to select the infected cells. In order to assess the functional differences between WT and ΔDOM Ikaros mutant we checked daily the GFP fluorescence by FACS on living cells (Figure 19-A, B). The puromycin treatment selects the cells that contain the viral construct, while the GFP analysis on living cells shows the percentage of GFP positive cells that express the protein of interest. The cells infected with the empty vector were successfully selected over 6 days of puromycin treatment – day 0 (61.85%) and day 6 (89.85%) GFP positive cells. As expected, WT Ikaros infected cells showed no enrichment of GFP positive cell after 6 days of puromycin treatment because of its repressive effect on the cell proliferation - day 0 (49 %) and day 6 (6.49 %). Surprisingly, the same effect can be seen in the ΔDOM Ikaros mutant, which apparently retains all the repressive properties of the WT protein - day 0 (42.2 %) and (8.65 %) at day 6. Unexpectedly, the percentage of GFP negative living cells after 6 days of puromycin treatment doubled in the WT and also increased in the the ΔDOM Ikaros mutant. This result suggests a loss of Ikaros expression with retained puromycin resistance during the drug treatment. The promoters driving the expression of Ikaros and the gene of resistance are in the same retroviral construct, a fact that suggests selective silencing of Ikaros expression during the selection period. In fact, an epigenetic event like methylation of viral LTRs (Long Terminal Repeats) in mammalian cells may cause silencing of the expression of the proviral genes (Svoboda et al., 2000; Swindle et al., 2004). In my experiment the same effect of silencing cannot be seen in the empty vector infected cells, because they maintain high expression of both GFP and the selection marker throughout the selection. This result is a technical drawback in studying the functional significance of the DOM region of Ikaros because of the inability of the generated cell line to both proliferate and maintain stable expression of this

mutant. This outcome is most probably due to the retained repressive ability of the ΔDOM Ikaros mutant on the cell proliferation. The proliferation of ILC87c Ikaros null T-cell line is due namely to the absence of Ikaros and its repressive effect on key proliferating genes. We therefore decided to change the target T-cell line with a T-cell line, the proliferation of which is Ikaros independent. The T-cell line 149.3B originates from a T-cell leukemia of a mouse strain with expression of constitutively active form of beta catenin (Kirstetter et al., 2006). In this mouse, the coding sequence of myc-tagged mutant beta catenin form (S33Y) is knocked in the ubiquitiously expressed R26 locus. This mutant beta catenin form cannot undergo phosphorylation and subsequent degradation, which makes this signaling pathway constitutively active and renders the T-cells hyper proliferative in the presence of endogenous Ikaros protein. Assuming that endogenous Ikaros protein is present in this cell line and its presence does not impair the cell proliferation, I infected the cells with the same viral particles (Figure 20-A, B). The efficiency of infection for this T-cell line with the empty vector was much lower – 12.5 % compared to ILC87c line – 61.85 %. Even though the initial low number of infected cells, the puromycin treatment efficiently selected high GFP expressing cells at day 6. That was not the case for WT or ΔDOM Ikaros mutant, which showed no GFP positive cells at day 6 after puromycin treatment. Similarly to the situation in ILC87c cell line, the cells were proliferating in the presence of puromycin without expression of GFP. This results led us to the conclusion that overexpression of Ikaros represses the cell proliferation even in a T-cell line that expresses endogenous Ikaros protein. The strong repressive effect of Ikaros on the cell proliferation doesn't allow the generation of a T-cell line overexpressing this protein in a constitutive manner. Thus, for further studies we decided to use a 4-hydroxytamoxifen (4-OHT) inducible system in which the protein of interest is overexpressed as an estrogen receptor ligand binding domain N-terminal fusion. This fusion protein accumulates in the cytosol and translocates in the nucleus upon 4-OHT treatment.

MDVDEGQDMSQVSGKESPPVSDTPDEGDEPMPVPEDLSTTSGAQQNSKSDRGMASNVKVETQSDEENGR
ACEMNGEECAEDLRMLDASGEKMNGSHRDQGSSALSGVGGIRLPNGKLKCDICGIVCIGPNVLMVHKRSHTGERPFQCN
QCGASFTQKGNLLRHIKLHSGEKPFKCHLCNYACRRRDALTGHLRTHSVGKPHKCGYCGRSYKQRSSLEEHKERCHNYL
ESMGLPGMYPVIKEETNHNEMAEDLCKIGA***ERSLVLDRLASNVAKRKSSMPQKFLGDK***CLSDMPYDSAN***YEKEDMMTSH***
VMDQAINNAINYLGAESLRPLVQTPPGSSEVVPVISSMYQLHKPPSDGPPRSNHSAQDAVDNLLLLSKAKSVSS***EREAS***
PSNSCQDSTDTESNAEEQRSGLIYLTNHINPHARNGLALKEEQRAYEVLRAASENSQDAFRVVSTSGEQLKVYKCEHCR
VLFLDHVMYTIHMGCHGFRDPFECNMCGYHSQDRYEFSSHITRGEHRYHLS

Figure 18. Schematic representation of the DOM deleted region of Ikaros (IK-ΔDOM)

The amino acid sequence of Ikaros protein (515 amino acids) is represented by black letters. The cysteine and hystidine residues of N- and C- terminal Zn fingers are plotted as follows with black or red color. Ikaros activation domain is underlined, while the three conserved across the Ikaros family motifs are shown in bold *italic* shrift. The size of the deleted DOM region (147 amino acids) is shown in yellow.

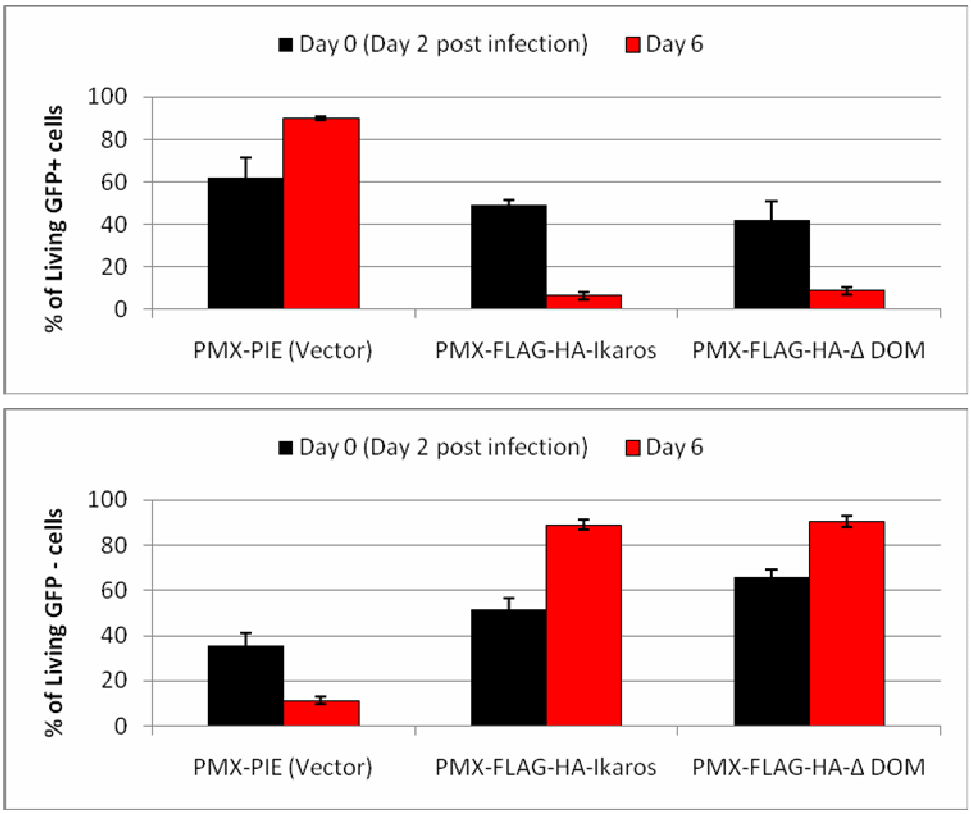


Figure 19-A. Overexpression of WT and ΔDOM Ikaros in ILC87c IK-/- cell line after 6 days of puromycin treatment

ILC87c cell line has been infected with virus particles containing the empty vector PMX-PIE, WT or ΔDOM Ikaros tagged with FLAG-HA. At day 0 (black bars) the cells are plated in medium containing puromycin (1ug/ml) and cultured for six days (Day 6 red bars). The medium was changed daily with fresh one supplemented with antibiotic. The percentage of GFP positive and negative cells is estimated daily by FACS.

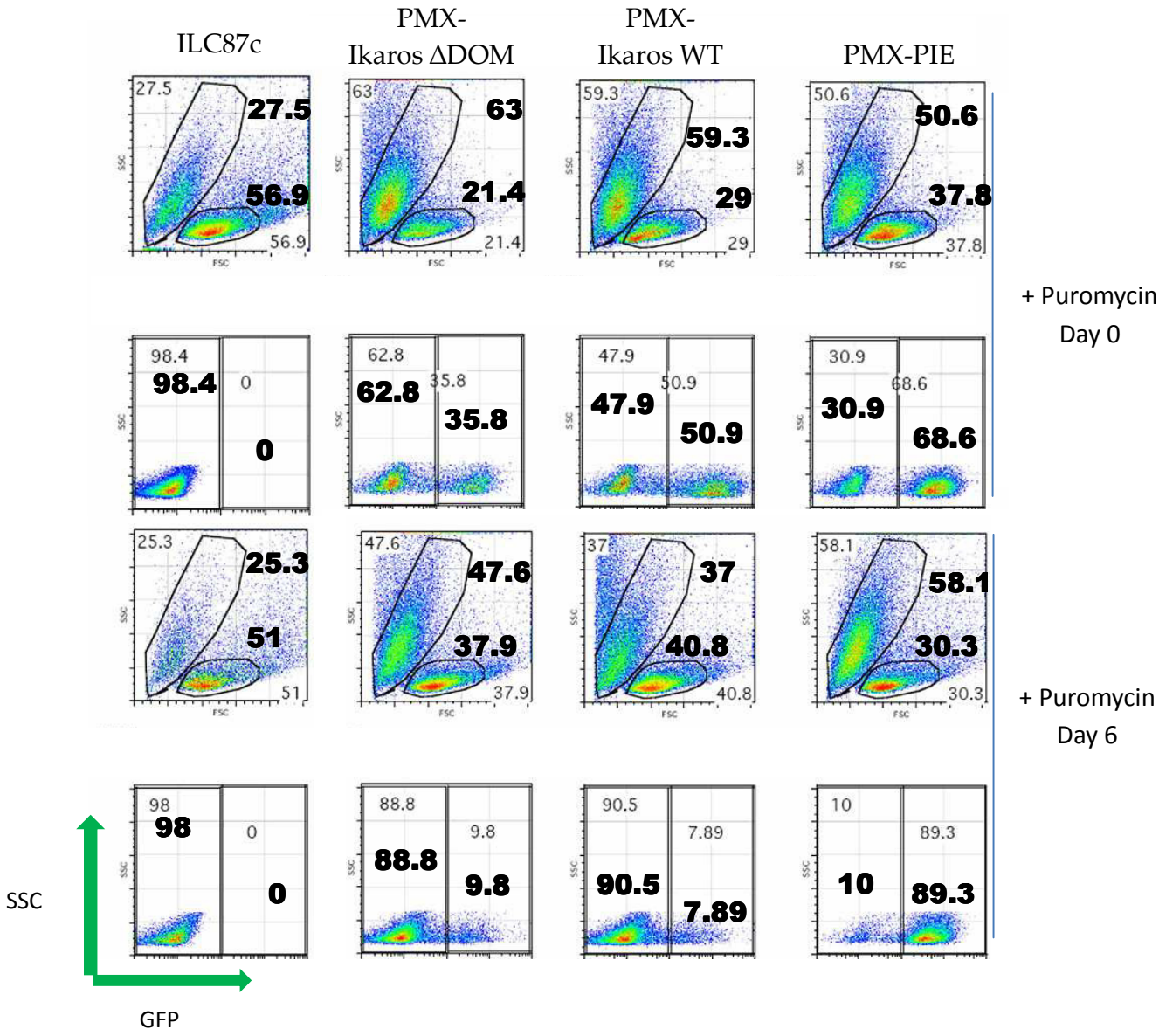


Figure 19-B. Overexpression of WT and ΔDOM Ikaros in ILC87c cell line after 6 days of puromycin treatment (FACS plots).

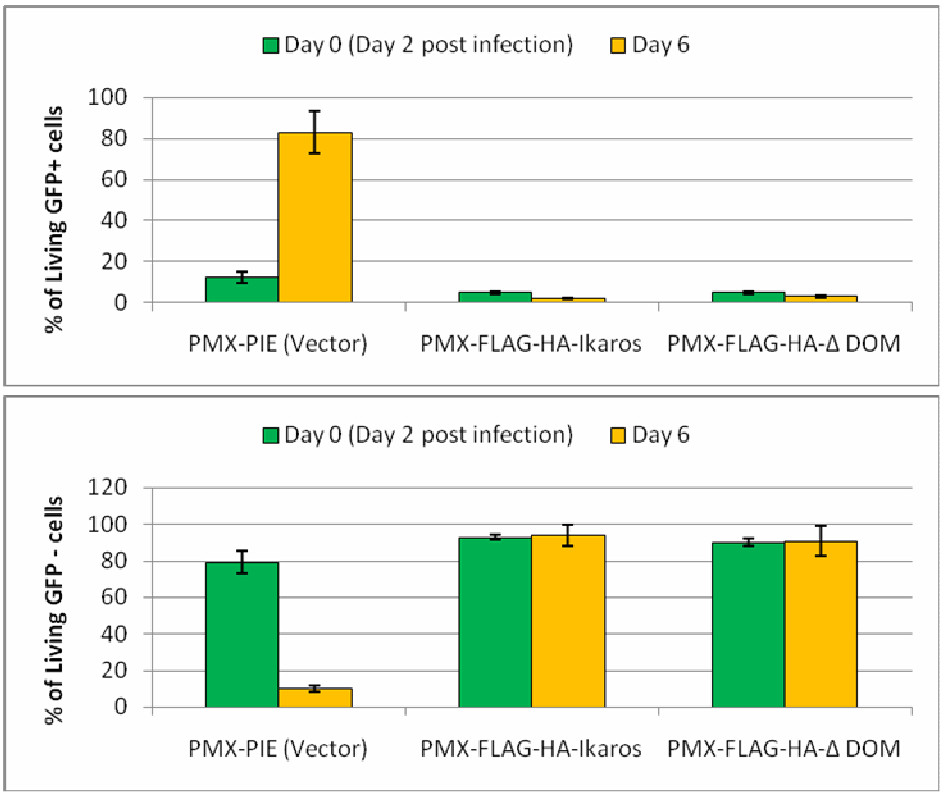


Figure 20-A. Overexpression of WT and ΔDOM Ikaros in 149.3B IK+/+ cell line after 6 days of puromycin treatment

149.3B IK+/+ cell line has been infected with virus particles containing the empty vector PMX-PIE, WT or ΔDOM Ikaros tagged with FLAG-HA. At day 0 (green bars) the cells are plated in medium containing puromycin (1ug/ml) and cultured for six days (Day 6 orange bars). The medium was changed daily with fresh one supplemented with antibiotic. The percentage of GFP positive and negative cells is estimated daily by FACS.

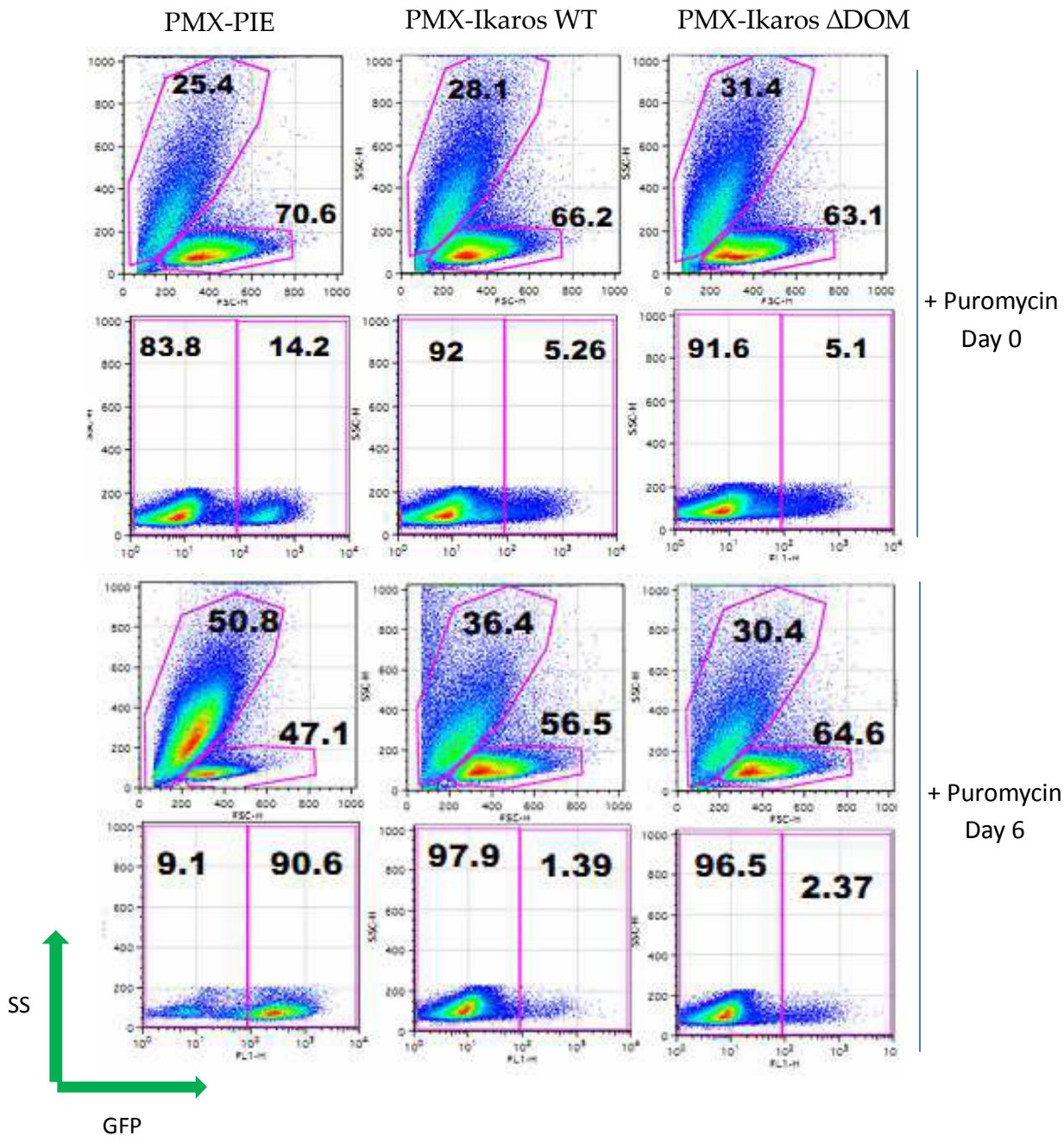


Figure 20-B. Overexpression of WT and ΔDOM Ikaros in 149.3B IK+/+ cell line after 6 days of puromycin treatment (FACS plots).

III. SECOND TOPIC

III.2 Studying the posttranslational modifications of transcription factor Ikaros and their role in its function.

III.3 Background studies

The main topic of my PhD was to study the post-translational modifications of transcription factor Ikaros and their role in its function. Up to now, only one study addressed this question (Gómez-del Arco et al., 2005). Their results show that Ikaros is sumoylated in total primary T-cells, a dynamic event that modulates its repressive abilities. They describe two consensus sumoylation sites on Ikaros (K58 and K240), the sumoylation of which leads to loss of Ikaros repressive function in ectopic reporter gene assays. The final conclusion of the study is that sumoylation does not alter the nuclear localization of Ikaros but acts as a mechanism disrupting its participation in both histone deacetylase (HDAC) dependent and independent repression. The experiments described above were performed in cell types like 293T, 3T3 NIH and U2 OS that do not express endogenous Ikaros protein. This is an advantage regarding the cytotoxicity of Ikaros when overexpressed in T-cell lines, but the ectopic expression may not be the most relevant model for studying post-translational modifications. The nature of the these protein modifications is usually transient and dependent on a number of factors including the differentiation stage (Kwon et al., 2012), cell cycle progression (Bakiri et al., 2000) or DNA damage events like genotoxic stress (Feng et al., 2005). Thus, we decided to address the Ikaros sumoylation in more detail in a relevant model system such as Ikaros deficient T-cell line.

III.4 Aim of the study

My work is focused mainly on the sumoylation of Ikaros, a field less explored and contradictory up to the present moment. The presence of more, un-described sumo-modified Ikaros fractions in our experiments, compared to the previous report, led us to investigate this process in more

detail. To identify the additional SUMO sites on Ikaros I used two main approaches. The first is to introduce deletions or point mutations lysine (K) to arginine (R) in the IK1-ER coding sequence that will directly map the un-described sumo acceptor sites. This led to the generation of a library of cell lines expressing IK1-ER mutants. They were tested for the absence of modified fractions by immunoprecipitation and western blot assays. The proliferation of the mutant cell lines was also tested in order to reveal functional differences among the mutants. The second strategy was to concentrate the modified fractions and to study them by mass-spectrometry. I also established cell lines (using the same ILC87c Ikaros null T-cell line) that overexpress the others members of Ikaros family – Helios, Aiolos and Eos as ER fusions in order to compare their sumoylation profile with that of Ikaros.

III.5 Posttranslational modification of Ikaros during T-cell development

The T-cell development is characterized by different stages, as the most immature cells are double negative for the expression of CD4 and CD8. Western blot on the double negative fraction 3 and 4 (DN3 and DN4) showed different patterns of Ikaros modification compared to the double positive (DP) stage (Kleinmann et al., 2008) (Figure 21.A).

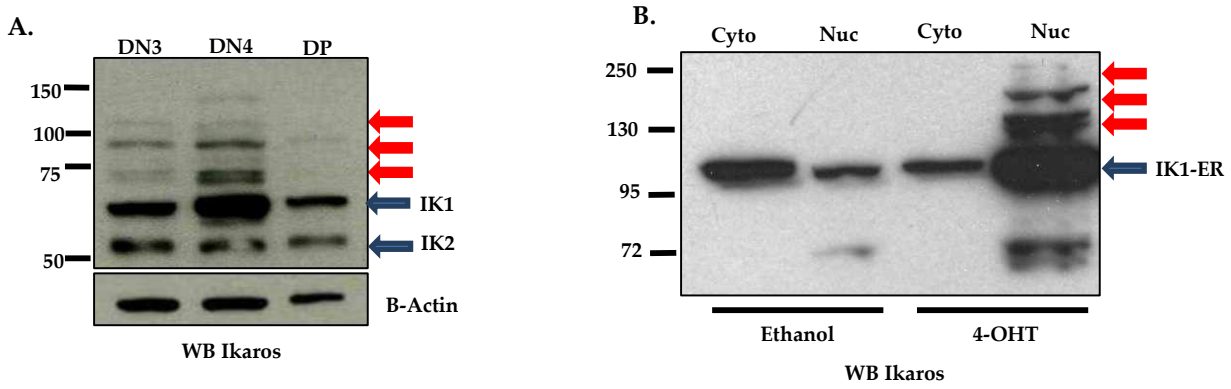


Figure 21. Ikaros is posttranslationally modified in primary T-cells during T-cell development as well as in T-cell line overexpressing an inducible Ikaros-ER fusion.

(**A**). Total thymocytes from wild type 6 weeks old mouse were sorted and 5 x 10^5 DN3, DN4 or DP cells were lysed in 1x loading buffer, run on a 8 % SDS-polyacrylamide gel, transferred on PVDF membrane and probed with anti Ikaros antibody. Beta actin is shown as a loading control. The red arrows show the modified fractions, blue arrows - unmodified Ikaros 1 and Ikaros 2 isoforms. (**B**). Cytosolic and nuclear fractions corresponding to 1 million cells, from

Ethanol or 4-OHT (100nM) treated Ikaros-ER overexpressing ILC87c cells were boiled in 1x loading buffer and subjected to SDS-PAGE on 10% gel, transferred to a PVDF membrane and probed with anti Ikaros antibody.

The amount of the modified protein is not only higher during DN stage compared to DP, but a difference can be seen even between the DN3 and DN4 stages. In DN4 stage, up to six high molecular weight bands with different intensities can be detected, a profile that is less obvious in DN3 and especially in the DP fraction. This fact outlines the importance of the development stage for the complete posttranslational profile of Ikaros. Ikaros is sumoylated in vivo (Gómez-del Arco et al., 2005) but the nature of this modification hasn't been clearly demonstrated in wild type thymocytes. The main technical drawback in studying posttranslational modifications in primary double negative cells is the limited amount of this cell fraction – only 2 to 3 % of the total thymocytes, and the complexity and the costs of the process of enrichment of double negative cells. Moreover, the amount of the modified protein is only a small fraction of the endogenous total unmodified pool of Ikaros. All together, these technical restraints make the characterization of the post transnationally modified Ikaros fractions in primary cells very difficult. Thus, we decided to address this question in a T-cell line, which is easy to handle and gives a higher amount of material. On the other hand, overexpression of Ikaros in T-cell lines leads to inhibition of proliferation and cell death (Dumortier et al., 2006). Therefore we decided to use a system in which Ikaros isoform 1 is overexpressed as an estrogen receptor ligand binding domain fusion – IK1-ER. Thus, for my experiments, I used an Ikaros null T-cell line ILC87c, obtained from a T-cell leukemia of a Ikaros fl/fl x Lck-Cre^{+} mouse line. This cell line was described as a part of my first topic the aim of which was to characterize the transcription activation domain of Ikaros. After infection of ILC87c cells with retrovirus harboring the IK1-ER cDNA, Ikaros is constitutively overexpressed in the cytosol as an ER fusion protein and undergoes translocation to the nucleus upon 4-OHT treatment (Figure 21.B). Ethanol treatment doesn't trigger nuclear translocation suggesting that the small amount of the protein in the nucleus in the ethanol treated control may be a result of the "leakiness" of this model system or because of contamination with small amount of nuclear proteins. Nevertheless, this approach overcomes Ikaros cytotoxicity and allows studying its posttranslational status because of the presence of modified Ikaros fractions similar to those in primary DN cells.

III.6 Optimizing the conditions for efficient protection of the modified Ikaros fractions during the cell lysis.

For successful studying the modified Ikaros fractions the cell lysis step should be optimized to protect the complete pattern of sumoylated Ikaros. In the experiments shown in Figure 21, the cell lysis is performed by boiling intact cells or nuclear pellets in loading buffer. The composition of this buffer is not compatible with any functional assay such as immunoprecipitation (IP) which is necessary for enrichment the modified fractions and their subsequent analysis. The presence of high amounts of denaturing agents like 1.5 - 2% SDS (sodium dodecyl sulphate) and reducing agents like 100 mM DTT (Dithiothreitol) or 1.5-2 % Betamercaptoethanol (BME) makes the lysate convenient only for western blot assays. Thus, I started the optimization of the lysis step by dialyzing the lysates with 1 kD cutoff dialyzing membrane. This process leads to efficient cleaning of the reducing agents because they are soluble at 4^{o}C, but inefficient dialysis of the SDS. At 4^{o}C this compound forms crystals that cannot pass through the membrane pores, which leads to subsequent unsuccessful immunoprecipitation (data not shown).

The attempt to dialyze the lysate at 15^{o}C was successful, but at this temperature the overall stability of the proteins and especially the modified fractions is very low, resulting in very weak recovery after IP. In their pioneering study of Ikaros sumoylation Gómez-del Arco and colleagues used N-ethylmaleimide as a de-sumoylation inhibitor (Figure 22). The process of sumo conjugation and de-conjugation is dynamic and performed by enzymes belonging to the class of iso-peptidases (Melchior et al., 2003), the action of which can be inhibited by N-ethylmaleimide (Lyst et al., 2006; Gómez-del Arco et al., 2005; Ungureanu et al., 2003). Using this alkylating agent as a de-sumoylation inhibitor we detected sumoylated Ikaros bands after ectopic co-expression of Ikaros and HA-SUMO2 in 293T cells (Figure 23-A), suggesting that Ikaros might also be target for SUMO-2/3 conjugation. The number of bands seen in this 293T cell experiment was lower compared to DN4 thymocytes or IK1-ER expressing T-cell line, which prompted us to investigate this process in a T-cell context rather than in irrelevant cell types. The presence of several modified Ikaros fractions in T-cells was not reported up to the present moment. It is therefore possible that Ikaros is targeted to modification by ubiquitin or other ubiquitin-like proteins in addition to its sumoylation.

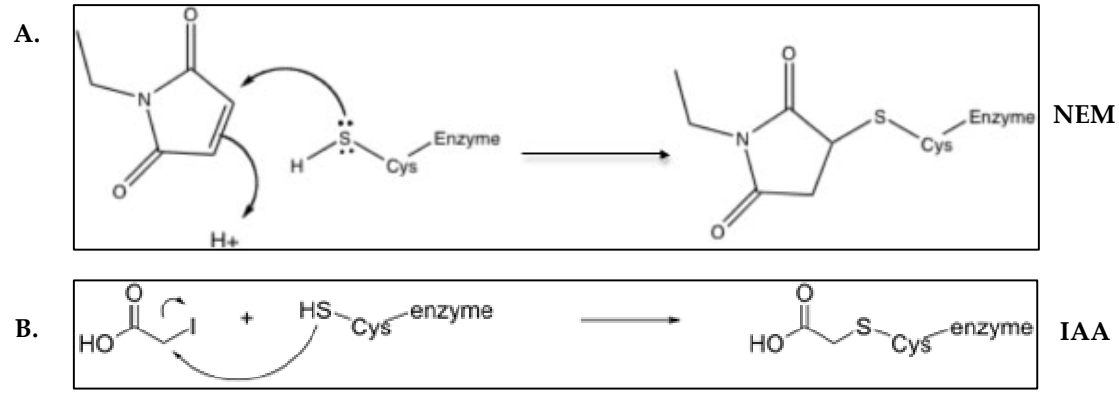


Figure 22. Structure of N-ethylmalemide (NEM), Iodoacetic Acid (IAA) and the mechanism of irreversible inhibition of cysteine peptidases by these two compounds.

(**A**). NEM is an organic compound derived from maleic acid which contains an imide functional group. It is reactive toward thiol groups which makes it commonly used to modify cysteine residues in proteins and peptides. The resulting C-S bond is strong and the reaction is virtually irreversible. Reaction with thiol groups occurs in the pH range (6.5–7.5). (**B**) Iodoacetic acid is an inhibitor of all cysteine peptidases, with a mechanism of inhibition similar to NEM. Both NEM and IAA are irreversible inhibitors of cysteine peptidases leading to alkylation of the active site thiol group.

Moreover, there are 17 known ubiquitin-like proteins (UBLs) like NEDD8, SUMO1,2,3,4, ISG15, FUB1, FAT10, Atg8, Atg12, Urm1, UFM1, ThiS and MoaD that have been identified to be conjugated to substrates in a manner analogous to ubiquitin (Kerscher et al., 2006; Hochstrasser M., 2000). In fact, it has been already published that Ikaros is ubiquitinated in human (MOLT-4) and murine (VL3-3M2) leukemia T-cell lines, an event that leads to its degradation mediated by ubiquitin/proteasome pathway (Popescu et al., 2009). We therefore decided to use NEM in combination with second iso-peptidase inhibitor – Iodoacetic acid (IAA), a well-known de-ubiquitination and de-neddylation inhibitor (Xirodimas et al., 2004). The higher reactivity and inhibitory effect of IAA is due to the interaction between the positive imidazolium ion of the catalytic histidine on the enzyme and the negatively charged carboxyl-group of the Iodoacetate (Polgar, L., 1979). Thus, I performed total cell lysis of IK1-ER expressing ILC87c cells upon 4-OHT treatment under denaturing conditions (1% SDS). The ability of both NEM and IAA to protect the sumoylated fractions separately and in combination was tested. Their inhibitory effect was also assessed in the presence of strong reducing agent as 20 mM DTT (Figure 23-B). Unexpectedly, these two inhibitors used separately resulted in a different modified profiles with an obvious shift of the major modified bands (Figure 23-B) lanes (N = NEM) and (I =IAA). When used together -

lane (N+I) we detected the complete modified profile of Ikaros similar to primary T-cells. Interestingly, the presence of DTT in the lysis buffer resulted in a loss of the minor modified fractions, showing the incompatibility of high doses of reducing agents and thiol group-alkylating reagents like NEM and IAA. Taken together these results suggest that Ikaros is posttranslationally modified on more than two acceptor sites. On the other hand, the fact that a combination of two different inhibitors protects the complete sumoylation profile suggests a different nature of these modified fractions.

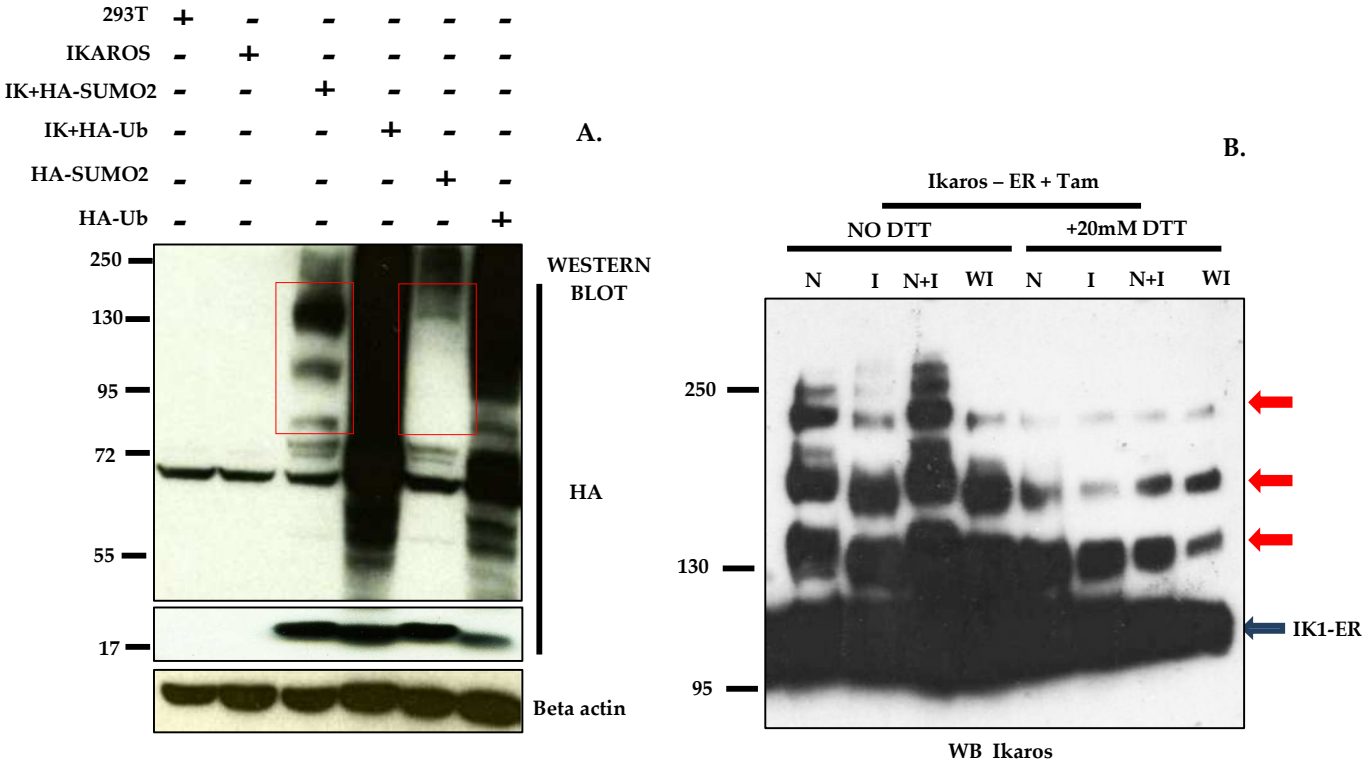


Figure 23. Ikaros is postranslationaly modified by Ubiquitin-like proteins in 293T cells and Ikaros null T- cell line.

(**A**) 293T cells transfected with Ikaros, HA-SUMO2, HA-Ubiquitin alone or in combination with Ikaros are lysed in buffer containing 10 mM NEM. Total cell extracts equivalent to 2 million cells were subjected to PAGE on a 15 % gel, transferred to a PVDF membrane and probed with an anti HA antibody. Beta actin is shown as a loading control. (**B**) ILC87c cells overexpressing IK1-ER were treated for 24 h with 100 nM 4-OHT and lysed in denaturing buffer containing 1% NP-40 and 1% SDS in presence, absence or combination of de-sumoylation inhibitors - 10 mM NEM and 10 mM IAA. The efficiency of these inhibitors is tested in the absence or presence of 20 mM the reducing agent DTT (Dithiothreitol). Total cell extracts equivalent to 2 million cells were subjected to PAGE on a 6 % gel, transferred to PVDF membrane and probed with anti Ikaros antibody. N = NEM (N-Ethylmaleimide); I = IAA (Iodacetic acid); WI = Without Inhibitors.

Since Ikaros expression level is higher during DN4 stage, while its mRNA doesn't show substantial change (data not shown), we next wanted to assess whether Ikaros undergoes other type of modification such as ubiquitination, an event that could potentially modulate its stability. Conditional, DNA damage dependent for p53 (Toledo et al., 2007) or stage-dependent poly-ubiquitination of a transcription factors like E2A (Sun L., 2007), c-Jun/AP1 and IκB-α/NF-κB (Gu et al., 2007) can lead to their degradation in proteasome dependent manner, while the attachment of mono-ubiquitin may have quite different effect on their properties, such as change in their subcellular localization and transcriptional activity (Brooks et al., 2004; Horst et al., 2006). Thus, to determine whether Ikaros is ubiquitinated in our model system, IK1-ER cell line was treated with 100 nM 4-OHT for 24 h and also with two more inhibitors - 26S proteasome inhibitor MG132 (20 uM) - 2 hours prior to lysis and the in vivo sumoylation inhibitor Anacardic acid (Fukuda et al. 2009) - (50 uM) - 24 hours prior to lysis .The cytosolic fraction was separated from the nuclear by incubating the cells with hypotonic buffer supplemented with 10 mM NEM and 10 mM IAA. The nuclear fraction was lysed in buffer containing 1 % NP-40 in the presence of - 10 mM NEM and 10 mM IAA. For each lane nuclear extracts equal to 50 million cells were subjected to IPs with ER antibody. After transferring the proteins on PVDF membrane, they were probed with antibodies against Ubiquitin and Ikaros (on the same membrane) or mixed SUMO1-2/3 antibodies (Figure 24). My results suggest that Ikaros is not mono- or polyubiquitinated because none of the detected with anti-Ikaros antibody bands is reactive for ubiquitin. The blocking of ubiquitin/26S proteasome pathway by incubation of the cells with MG132 doesn't lead to appearance of ubiquitinated bands or high molecular weight "smear" signal typical for the polyubiquitinated substrates. This treatment leads to minimal decrease of the amount of the modified fractions with small increase in the background signal in the IP lane (+MG132). Obviously, the signal does not overlap with Ikaros signal, which is most probably due to a small amount of ubiquitinated proteins which co-IP with Ikaros under native conditions in 300 mM NaCl. The last lane shows whether Ikaros may be ubiquitinated when its sumoylation is impaired. The Anacardic acid inhibits the sumoylation process in vivo impairing an intermediate step of this enzymatic cascade - formation of the complex E1 activating enzyme

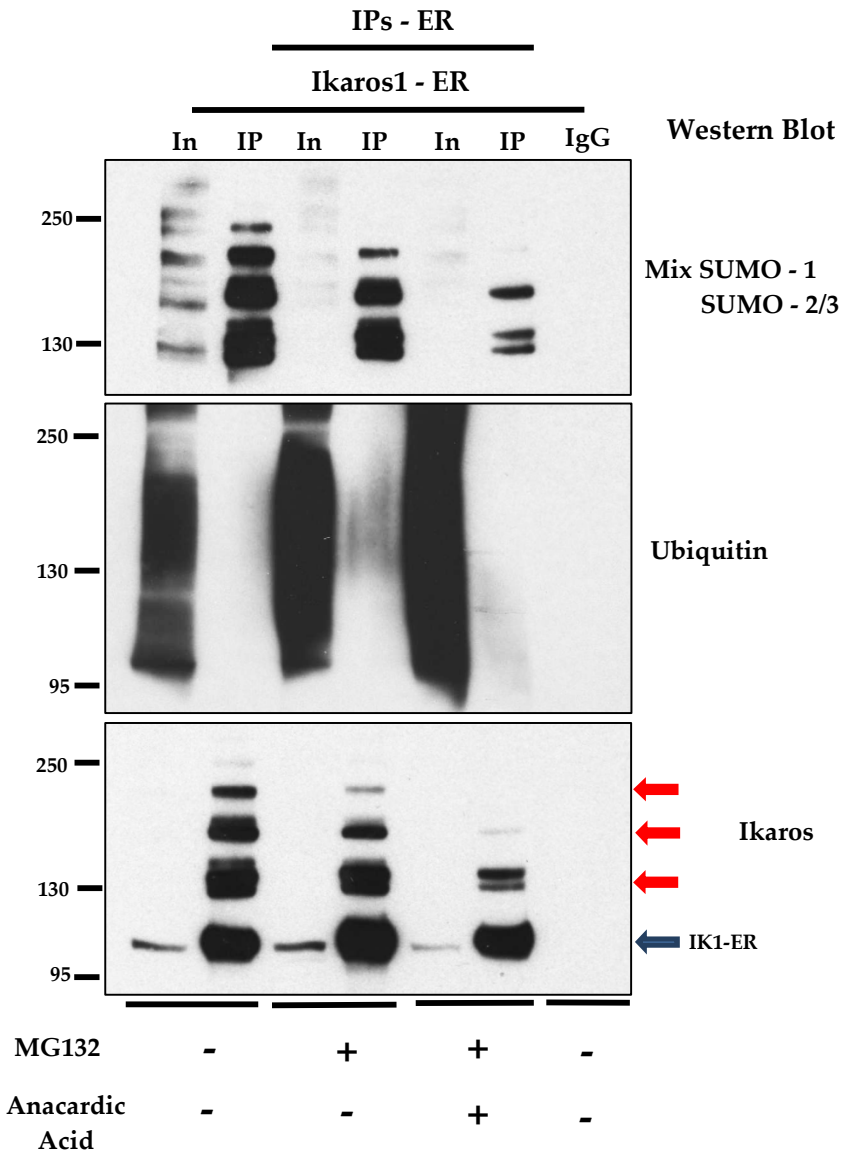


Figure 24. Ikaros is sumoylated by SUMO-1 and SUMO-2/3 but not Ubiquitinated in Ikaros null T- cell line.

ILC87c cells overexpressing IK1-ER was treated for 24 h with 100 nM 4-OHT in absence or presence of 26S proteasome inhibitor MG132 (20 uM – 2 hours) and/or the sumoylation inhibitor Anacardic acid (50 uM – 24 hours) and lysed in buffer containing 0.5 % NP-40 in the presence of de-sumoylation inhibitors - 10 mM NEM and 10 mM IAA. The nuclear extracts from 50 million cells were subjected to IPs with ER antibody bound to protein G beads. The beads were washed 3 times with lysis buffer and boiled in 3x sample buffer. The extracts were subjected to PAGE on 6 % gel, transferred to PVDF membrane and blotted with the indicated antibodies. In = Input (5%); IP = Immunoprecipitation; IgG = mouse IgG used as negative control for the IP on IK-ER nuclear extract

- SUMO (Fukuda et al. 2009). In my experiments I used this sumoylation inhibitor in order to assess a possible ubiquitination when the sumoylation process is impaired. The interplay between sumoylation and ubiquitination is well known. For example, Human T-Cell Leukemia Virus Tax oncoprotein is sumoylated and ubiquitinated on the same lysine residue, as this interplay mediates Tax regulated activation of Nuclear Factor kB (NFkB) (Lamsoul et al., 2005). Another example for the importance of this interplay for transcription factor function is the regulation of NFkB activity by its inhibitor IkB. IkB is both sumoylated and ubiquitinated on the same lysine residue involving this interplay in the modulation of IkB stability and thus NFkB activity (Desterro et al., 1998). Treatment with Anacardic acid treatment led to a significant reduction of the modified fractions, without significant changes of the unmodified Ikaros amount. Obviously, in this model system Ikaros is neither mono- nor polyubiquitinated even when its sumoylation is dramatically reduced. Taken together, all these results suggest that Ikaros is not ubiquitinated but sumoylated at more than two lysine residues, by more than one type of SUMO protein.

III.7 Mapping the sumoylated regions by inserting a deletions in IK1-ER sequence

A frequently used approach to reveal the sumoylated parts of one given protein is the insertion of deletions that lead to loss of modified fractions. This approach is useful in many, but not in all the cases. The loss of a band might be due to the loss of binding site for the modifying enzyme or its partners, misfolding (if the right folding is necessary for the modifying step), because of change of other properties of the protein itself, but not because of the loss of the modified lysine. Nevertheless, I introduced several deletions of different size at specific regions of Ikaros and overexpressed these delta (Δ) mutants (Figure 25) as ER fusions in the ILC87c cell line. As it can be seen in figure 26, IK1-ER and Δ4K mutant showed a similar profile, while the larger mutants like Δ123, ΔDBD, ΔDOM and ΔDIM showed significant difference regarding the modified fractions. The Δ123 mutant, which lacks the first three exons, shows 5 bands, suggesting that this region contains two sumoylation sites. This mutant cannot be seen on the bottom panel because it lacks the peptide recognized by the N-terminal antibody. Surprisingly, the ΔDBD mutant shows no modifications at all, suggesting an importance of the DNA binding ability of Ikaros for its successful modification by SUMO.

MDVDEGQDMSQVSGKESPPVSDTPDEGDEPMPVPEDLSTTSGAQQNSKSDRGMASNVKVETQSDEENGRACEMNGEECAEDLRMLDASGEKMNGSHRDQ
GSSALSGVGGIRLPNGKLKCDICGIVCIGPNVLMVHKRSHTGERPFQCNQCGASFTQKGNLLRHIKLHSGEKPFKCHLCNYACRRRDALTGHLRTHSVG
KPHKCGYCGRSYKQRSSLEEHKERCHNYLESMGLPGMYPVIKEETNHNEMAEDLCKIGAERSLVLDRLASNVAKRKSSMPQKFLGDKCLSDMPYDSANY
EKEDMMTSHVMDQAINNAINYLGAESLRPLVQTPPGSSEVVPVISSMYQLHKPPSDGPPRSNHSAQDAVDNLLLLSKAKSVSSEREASPSNSCQDSTDT
ESNAEEQRSGLIYLTNHINPHARNGLALKEEQRAYEVLRAASENSQDAFRVVSTSGEQLKVYKCEHCRVLFLDHVMYTIHMGCHGFRDPFECNMCGYHS
QDRYEFSSHITRGEHRYHLS

Figure 25. Schematic representation of Ikaros showing deleted regions in different colors.

Yellow - Δ 123 (mutant which lacks the first three exons); Green - Δ DBD (mutant which lacks the DNA Binding Domain); Red - Δ DOM (mutant which lacks the transcription activation domain plus 3 conserved among Ikaros family parts); Pink - Δ4K (mutant included in the Δ DOM mutant); Light blue - Δ DIM (mutant which lacks the Dimerization Domain)

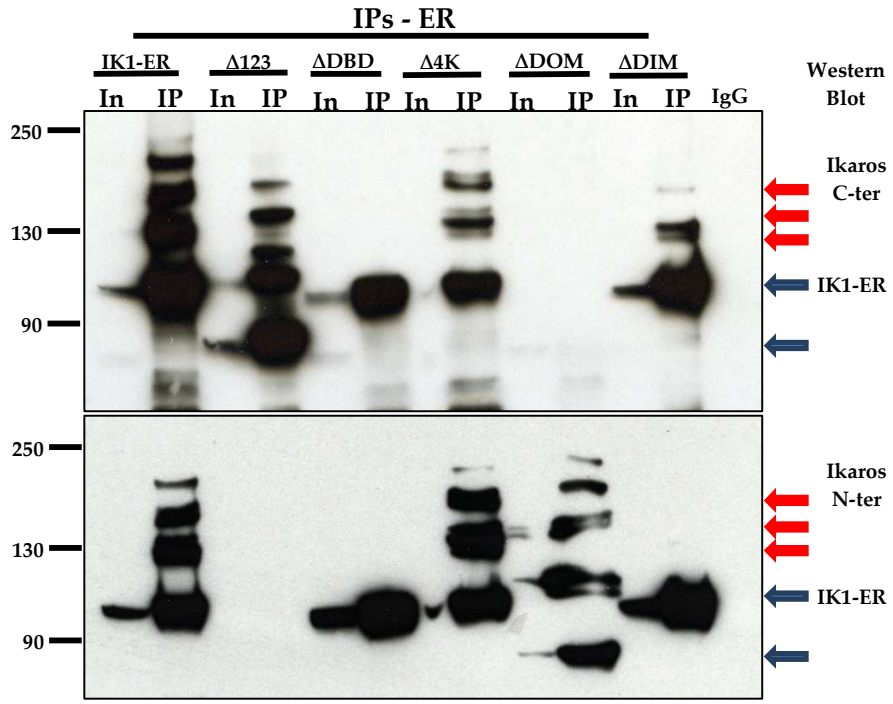


Figure 26. Different pattern of modified Ikaros in WT compared to N – and C – terminal deletion mutants.

ILC87c cells overexpressing IK1-ER and described above mutants was treated for 24 h with 100 nM 4-OHT and lysed in buffer containing 0.5 % NP-40 in the presence of de-sumoylation inhibitors - 10 mM NEM and 10 mM IAA. The nuclear extracts from 50 million cells were subjected to IPs with ER antibody bound to protein G beads. The extracts were subjected to PAGE on 6 % gel, transferred to PVDF membrane and blotted with C-terminal Ikaros antibody (top) and N – terminal (bottom). In = Input (5%); IP = Immunoprecipitation; IgG = mouse IgG used as negative control for the IP on IK-ER nuclear extract. The bottom panel represents the same membrane, stripped and blotted with an Ikaros antibody raised against N – terminal Ikaros peptide (H100).

The largest mutant ΔDOM shows a modified pattern very similar to WT Ikaros in term of the number of the modified bands. Note that, this mutant cannot be seen on the upper panel because it lacks the epitope recognized by the C-terminal antibody. Interestingly, the ΔDIM mutant which lacks the two C-terminal zinc fingers (the dimerization domain) shows a limited number of bands – three. Assuming that this part doesn't contain any lysine residues, we can speculate that a dimerization of Ikaros is required at least for some of the modified species. The complete absence of SUMO in the ΔDBD mutant or its reduction in ΔDIM mutant may have another explanation - these parts may contain a docking site that is important for the modification process. It is well known that the unique SUMO conjugating E2 enzyme - Ubc9 directly binds and sumoylates the consensus sumoylation motif (ϕ K x E/D), where ϕ can be a hydrophobic amino acid and x any amino acid. This fact doesn't exclude the existence of sites in the DBD and DIM domains that are important for the binding of E3 sumo-ligating enzymes that help Ubc9 during the sumoylation process. Taken together, these results show that Ikaros contains multiple sumoylated lysines that are situated out of the large DOM region. This fact led me to inspect more carefully Ikaros protein sequence for additional sumoylation sites presented on it (Figure 27).

Figure 27. Schematic representation of Ikaros protein sequence with depicted consensus sumoylation sites.

The different exons are presented by stretches of black or blue letters, the published sumoylated lysines (58 and 240) are highlighted in yellow (Gómez-del Arco et al., 2005). The sites K425 and 459 were described as non-sumoylated in the same study. The site K118 was not described at all. These new potential sites are highlighted in green. The

cysteine and histidine residues of DNA binding and dimerization zinc fingers are highlighted respectively in black or violet.

Interestingly, in the study performed with ectopically expressed Ikaros in 293T cells (Gómez-del Arco et al., 2005) the sites K425 and 459 were described as non sumoylated, while the site K118 was not described at all. This is most probably due to the minimal amount of the correspondent modified fractions in 293T cells as well as the absence of IAA in the lysis buffers, outlining the importance of relevant model system when studying posttranslational modifications. Thus, I decided to create point mutants K to R at the described positions, and to combine them in double and triple mutants. The positions of the sites 118 (consensus) and 459 (non-consensus but sumoylated on some proteins; Liu et al., 2012) are right in front of the N- and C- terminal zinc fingers, suggesting a similar functional outcome of these modifications. Thus, I combined these two sites in a mutant called Double Mutant at the Zinc Fingers or briefly DMZF. Unlike all the mutant constructs in which all the examined lysines are substituted with arginines, in the DMZF, the consensus site LKCD was mutated into MNCD instead of LRCD. The immediate position of the lysine 118 right in front of the first cysteine residue of the first DNA-binding zinc finger suggests an important structural function of this amino acid. We were worried that replacing this lysine with an arginine might change the conformation of the first zinc finger. Therefore, in order to keep the DNA-binding ability of this mutant, I replaced the leucine 117 (L) and the lysine 118 (K) with methionine (M) and asparagine (N), mimicking the sequence found in Aiolos, a family member with the same DNA-binding specificity. The second multiple mutant that I created was Triple Mutant (TM) in which the lysines 58/240/425 were changed into arginine and their sumoylation profile was compared to IK1-ER (Figure 27). As we can see on figure 28, IK1-ER shows the complete profile of seven modified bands positive for both SUMO-1 and SUMO-2/3. The DMZF mutant not only shows a loss of one band, resulting in six-band profile, but also demonstrates low abundance of the bi-sumoylated fractions, suggesting that the high molecular weight bands of Ikaros might be poly-sumoylated. Most probably the sumoylated lysine in this mutant is the consensus site K118 (LKCD) but not the non-consensus one K459 (YKCE). Interestingly, the triple mutant TM (58/240/425) shows only two bands out of seven (Ikaros western blot) suggesting the presence of non-consensus sumoylated sites. If we assume that DMZF loses only one band and add this mutation to the TM, then we will have four point mutations resulting in loss of six bands, which leads to the speculation that there are at least two

more sumoylation sites on Ikaros. In fact, sumoylation of inverted or entirely non-canonical sites has been already reported (Ivanov et al., 2007; Friedlander R., Melchior F., 2007; Matic et al., 2010). Ikaros sumoylation has been reported to decrease its repressive abilities by impairing its interaction with HDAC-containing and HDAC independent repressive complexes (Gómez-del Arco et al., 2005). Therefore, I checked the amount of two Ikaros co-IP partners, members of the NURD (Nucleosome Remodeling and Deacetylase Repressor Complex) complex – HDAC-1 and MTA-2 (Zhang et al., 1999). In case of HDAC-1, we see a slight enrichment in the DMZF and TM lanes, while MTA-2 is a bit higher only in the TM lane. However, I have to note that the intensity of the signal for these proteins in the inputs is also higher compared to IK1-ER inputs. Thus, it is difficult to make a reliable conclusion whether in our model system the sumoylation impairs Ikaros ability to interact with NURD components. As the evidences of sumoylation on alternative, non-canonical sites (Ren et al., 2009) and its synergistic interplay with phosphorylation are growing (Tremblay et al., 2008; Vanhatupa et al., 2008; Hietakangas et al., 2006) I decided to create an Ikaros map in which all the published phosphorylated residues are mapped, which would facilitate the search for potential non-canonical sumoylation sites (Figure 29-A).

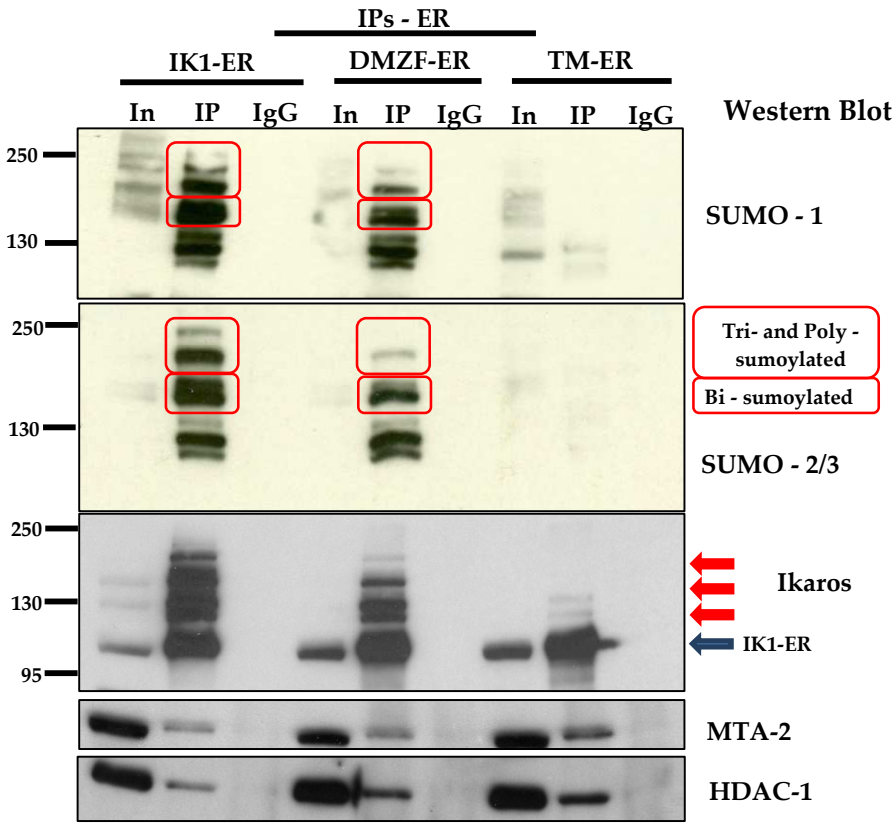


Figure 28. Ikaros is sumoylated by both SUMO-1 and SUMO-2/3.

ILC87c cells overexpressing IK1-ER, DMZF-ER and TM-ER were treated for 24 h with 100 nM 4-OHT and the nuclear fractions were lysed in buffer containing 0.5 % NP-40 in the presence of de-sumoylation inhibitors - 10 mM NEM and 10 mM IAA. The nuclear extracts from 50 million cells were subjected to IPs with ER antibody bound to protein G beads. The beads were washed 3 times with lysis buffer and boiled in 3x sample buffer. The extracts were subjected to PAGE on 6 % gel (for SUMO-1 and SUMO-2/3) or 4 – 12 % gradient gel (for Ikaros Western blot), transferred to PVDF membrane and blotted with the indicated antibodies. In = Input (5%); IP = Immunoprecipitation; IgG = mouse IgG used as negative control for the IP on IK1-ER nuclear extract.

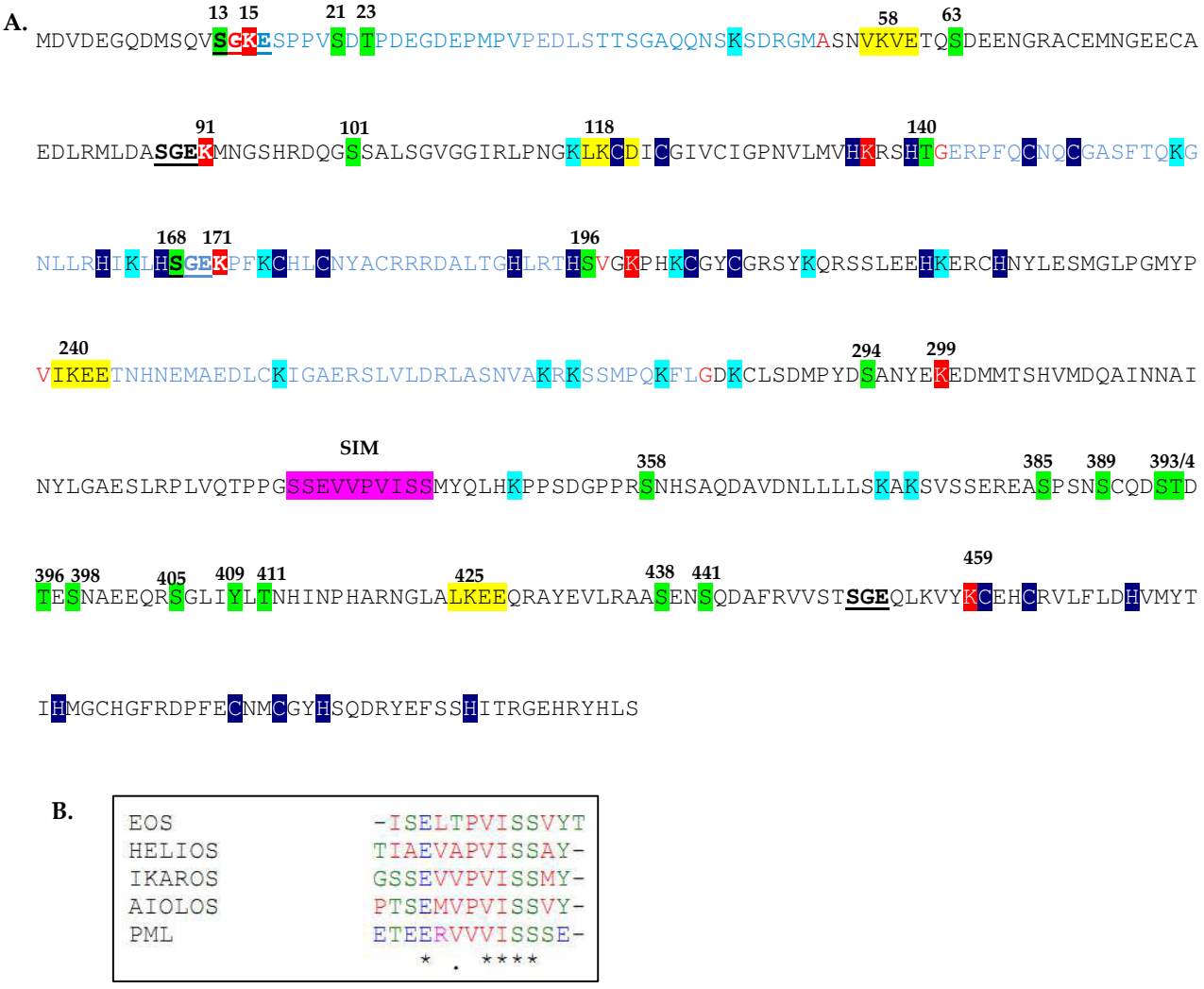


Figure 29. Schematic representation of Ikaros protein sequence with depicted phosphorylated and sumoylated sites as well as SUMO Interacting Motif (SIM) in its activation domain (Exon 7).

(**A**) The different exons are presented as stretches of black or blue letters, the sumoylated lysines (58, 118, 240 and 425) are highlighted in yellow. The published phosphorylation sites are highlighted in green. The cysteine and histidine residues of DNA binding and dimerization zinc fingers are highlighted in blue. The lysine residues in proximity to phosphorylated residues are shown in red. The repeated SGKE/SGEK motif is depicted with bold underlined shrift. All the resting lysines are highlighted in light-blue color. (**B**) Ikaros and Ikaros family members contain Sumo Interacting Motif (SIM) similar to the SIM of promyelocytic leukemia protein (PML).

Figure 7 (**A**) Ref: (Gómez-del Arco et al., 2005) – (S63, 385, 389, 394); (Gurel et al., 2008) – (S13, S101, 294, T23); (Sridharan R., Smale T., 2007) - (S63, 168, 358, 377, 385, 405, Y409, T394, 396, 411, 438, 441); (Popescu et al., 2009) - (S21, 389, 393, 398, T394, 396); (Dovat et al., 2002) – (S140, 168, 196);

III.8 Ikaros family members contain Sumo Interacting Motif (SIM)

An interesting observation is the presence of a putative SUMO Interacting Motif (SIM) on Ikaros and the other members of Ikaros family with the same DNA binding specificity (Figure 29-B). This motif and its environment are not strictly canonical and show diversity among the different proteins that contain it. It gives the ability of a given protein to interact with its partners only when they are sumoylated (Figure 30) (Hecker et al., 2006). Generally, this motif represents a short stretch of hydrophobic amino acids (Song et al., 2004; 2005; Hecker et al., 2006), flanked by acidic residues or residues that can be phosphorylated, a modification that increases its acidity (Stehmeier et al., 2009) and affinity to SUMO family members (Chang et al., 2011).

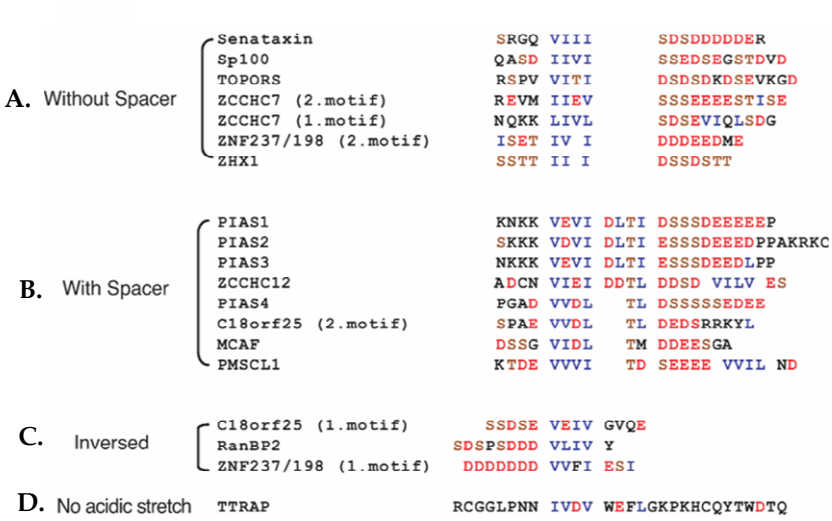


Figure 30. Alignment of different SUMO Interacting Motifs (SIMs) (reproduced from Hecker et al., 2006).

The SUMO Interacting Motifs can be heterogeneous in term of their environment. In some cases, the consensus hydrophobic core is flanked by acidic stretch and amino acids that can be phosphorylated. The main motif may contain or not a spacer sequence (**A, B**), to be in a reverse configuration (**C**) or without acidic environment (**D**).

III.9 Revealing the complete sumoylation pattern of Ikaros

The next step towards the uncovering the non-consensus sumoylation sites was to map the lysine residues (shown in red) nearby the phosphorylated residues (shown in green) – figure 29-A. I found an interesting motif - SGKE/SGEK, repeated three times throughout the Ikaros sequence - lysines 15, 91, 171 and slightly degenerate in front of lysine 456. Thus, I mutated the lysines - 15, 91, 171, as well 299 (because its proximity with phosphorylated tyrosine) into arginine and generated stable ILC87c cell lines overexpressing these point mutants. In addition, I created cell line expressing single point mutants on the consensus sumoylation sites, as well a cell line expressing the multiple 5KR mutant (58, 118, 240, 425 and 459) in which all the five lysines were converted into arginine. After doing IPs on nuclear extracts from these cell lines, I loaded the immunoprecipitated Ikaros on a 4-8% gradient gel in order to compare their sumoylation status and their ability to interact with the NURD component MTA-2 (Figure 31-A). As it can be seen, not all the mutants show a loss of sumoylated fractions. The mutants - 15, 91, 171, 299 and 459 show no difference compared to IK1-ER, confirming the absence of sumoylation at these positions. As mentioned above, Ikaros contains four consensus sumoylation sites but shows more than four modified fractions. The complete profile of seven bands can be seen only after longer exposure times of the film. This fact suggests the presence of non consensus sumoylation sites which might be dependent on the sumoylation of the canonical ones. As expected, the single point mutants on a consensus sites like 58, 240 and 425, as well the double, triple and the 5KR mutants demonstrate a loss of the sumoylated fractions to a different degree. Unexpectedly, in each single point mutant we see a loss of two bands instead of just one suggesting a dependency of the sumoylation of the non-consensus sites on the consensus ones. K58 mutant shows loss of band number 1 and 4 (figure 31-A and B), K240 loses bands 2 and 4, while K425 loses bands number 3, 5 and 7, acquiring an additional band migrating between bands 4 and 6. The double mutant (DM) 58/240 shows the presence of five bands (see the right, overexposed panel). The minimal amount of three of these fractions makes them visible only after very long exposure times (see the right panel). As expected, the double mutant at the zinc fingers (DMZF) 118/459 shows the presence of six bands (loss of band number 7) and weaker sumoylation of bands 4, 5 and 6. The K459R single point mutant does not show sumo-loss (Figure 31, lane 459), we can conclude that the lost

sumoylated band in the DMZF mutant is situated at position 118 in the wild type protein. The triple mutant (TM) 58/240/425 shows the presence of only two bands, suggesting that the loss of these three sumoylation sites leads to loss of four bands in this mutant. On this figure they can be seen after long exposure times, reflecting the minimal amount of the modified Ikaros on these sites. In our experimental system the protein of interest is overexpressed and sumoylated by the endogenous sumoylation machinery, which minimizes the risk of irrelevant sumoylation. A weak point of the system is the extremely low amount of some of the modified Ikaros fractions, which make the analysis of the sumoylated pattern of these mutants difficult. In the lane of 5KR mutant in which lysines 58/118/240/425/459 were mutated, no bands can be seen. The unmodified Ikaros band of this mutant is less intense than the corresponding band of the other mutants which questions the efficiency of the IP of this mutant. In theory, this 5KR mutant should have one modified band because it contains four mutated lysines that are sumoylated in the wild type Ikaros and one that is not (459). Thus, the absence of this band is most probably due to lower amount of total immunoprecipitated Ikaros, resulting in lower amount of the modified protein. Interestingly, the shift between the first three mono-sumoylated bands (number 1, 2 and 3, see Figure 31-A and B) of wild type IK1-ER corresponds to larger size fractions, but not mono-sumoylated fractions. The difference in the mass of the sumo family is about 1 kDa (SUMO-1 = 11.56 kDa, SUMO-2 = 10.87 kDa and SUMO-3 = 12.43 kDa). Even though we assume that the first three mono-sumoylated fractions represent Ikaros modified by the three different sumo members, the slight difference of 1 kDa would not allow a visible separation in this high molecular range of the gel. This result suggests that the migration properties of the mono-sumoylated forms on a SDS-PAGE gel are probably dependent on the position of the sumoylated residue. The more close to the N – or the C – terminal end of the protein is the sumoylated lysine, the faster is the migration of the mono-modified band. Thus, the sumoylation on K425 seems to slow down the migration of this modified fraction, compared to sumoylation on K58 and K240. In fact, the correlation between the position of the sumoylated lysine and its migration pattern has been already published for the transcriptional repressor basic Kruppel-like factor 3 (BKLF) (Pedromo et al., 2004).

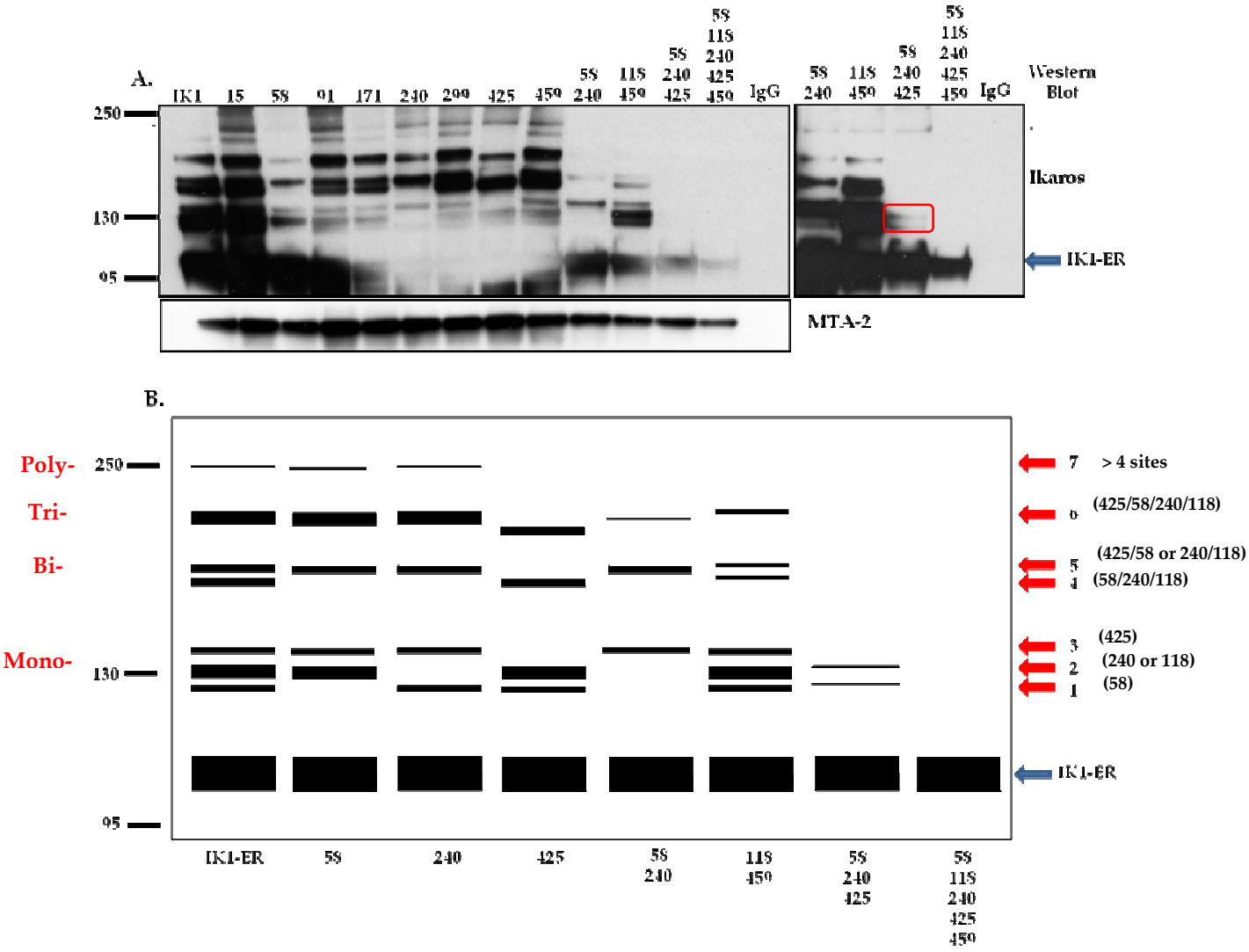


Figure 31. Sumoylation of WT and mutant Ikaros.

(**A**) ILC87c overexpressing IK1-ER, and the indicated mutants were treated for 24 h with 100 nM 4-OHT and the nuclear fractions were lysed in the presence of de-sumoylation inhibitors NEM and IAA. The nuclear extracts from 50 million cells were subjected to IPs with ER antibody bound to protein G beads. The extracts were subjected to PAGE on 4-8 % gradient gel, transferred to PVDF membrane and blotted with the indicated antibodies. The right part of the figure represents an overexposure of the same membrane. IgG = mouse IgG used as negative control for the IP on IK1-ER nuclear extract. The asterisk indicates an unspecific band. (**B**) Schematic representation and comparison of the modified fractions of Ikaros and the point mutants that show different sumoylation profile. Note that one visible band may contain more than one type of modified fraction. For example, the first three major bands would mask mono-sumoylated fractions at position 118, the amount of which is less abundant compared to the major ones.

In order to better separate the different sumoylated Ikaros fractions I run the immunoprecipitated Ikaros on 6 % gel (handling of PAGE gels less than 6 % is difficult because of their softness). For this experiment I used the same protein extracts used in figure 31, after one freeze-thaw cycle and I probed the three membranes with four different antibodies (Figure 32).

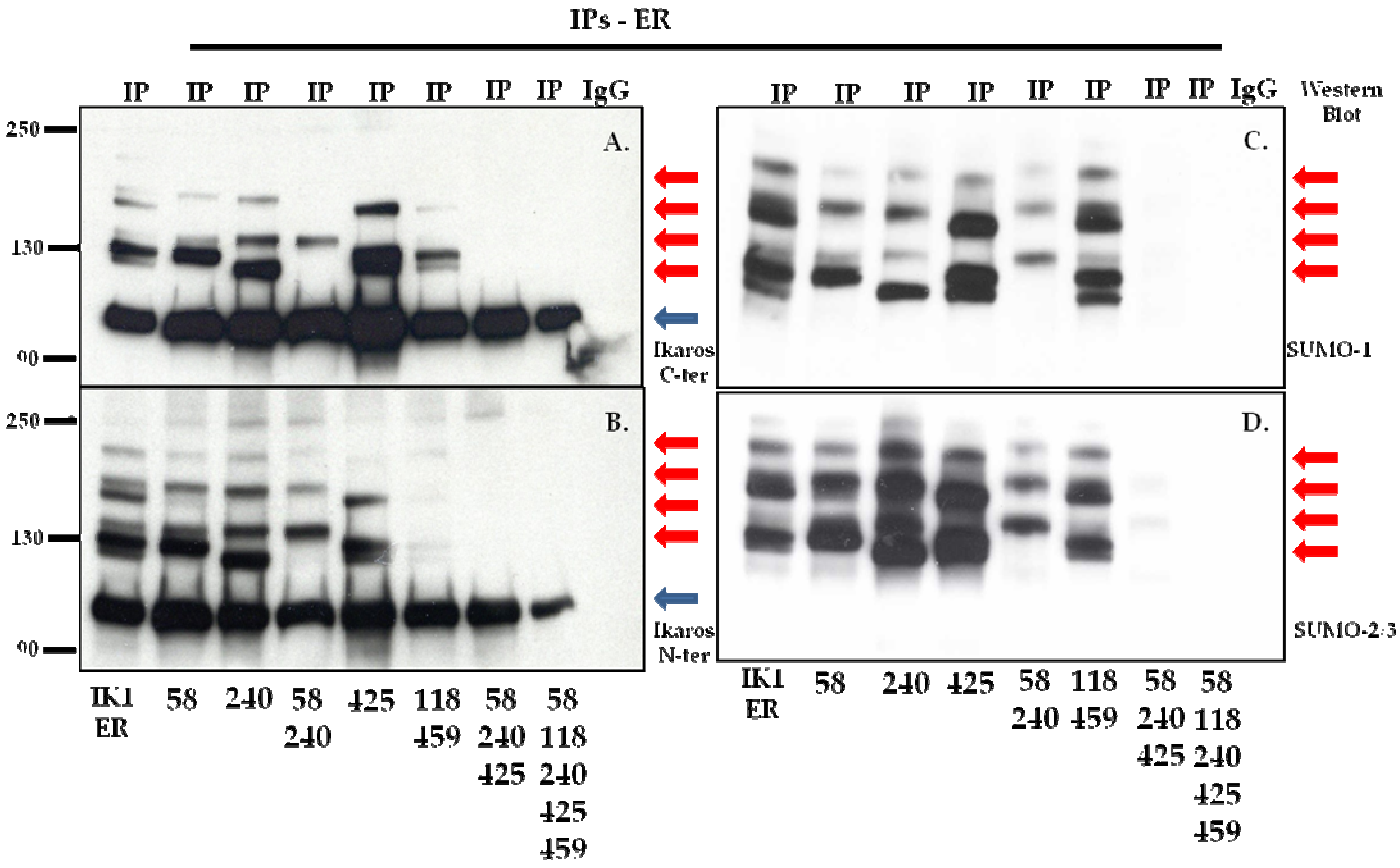


Figure 32. Separation of the sumoylated Ikaros fractions on 6% gel.

A protein extracts after one freeze-thaw cycle used in figure 31-A were subjected to IP with ER antibody and subsequent PAGE on 6 % gel, transferred to PVDF membrane and probed with four different antibodies: (**A**) with Ikaros C-ter., (**B**) the same membrane as in (**A**), stripped and reprobed with Ikaros N-ter. antibody, (**C**) with SUMO-1 and (**D**) with SUMO-2/3 antibody. IgG = mouse IgG used as negative control for the IP on IK1-ER nuclear extract. The asterisk indicates an unspecific band.

In this experiment, upon longer migration, I expected to see well separated modified Ikaros fractions but apparently the freeze-thaw step of the nuclear extracts leads to decrease of sumoylated Ikaros and subsequent weaker signal on western blot. Nevertheless, I was able to detect six modified fractions of IK1-ER with both Ikaros (N- and C- ter.) and SUMO-1 or SUMO-2/3 antibodies. This weak signal does not allow the appearance of the complete modified fractions in

the DM, DMZF and TM mutants when probed with the indicated antibodies (Figure 12). I cannot also exclude that the longer migration of the proteins in 6 % gel under low electric current may further decrease the overall protein amount by increased diffusion during migration. Interestingly, all the modified fractions of Ikaros are recognized by both SUMO-1 and SUMO-2/3 antibodies, which seems to be contradictory if we assume that each of these two antibodies is strictly specific for its target protein.

Taken together, these results lead to the characterization of additional, undescribed SUMO-acceptor sites on transcription factor Ikaros. It contains four consensus and at least one non-consensus SUMO acceptor sites. It seems that the attachment of SUMO to the non-consensus sites is dependent on the presence of sumoylated consensus lysines. It is difficult to estimate the exact number of the sumoylated fractions because of their minimal amount compared to the unmodified protein. The sumoylation process occurs in the nucleus and requires the DNA binding domain of Ikaros.

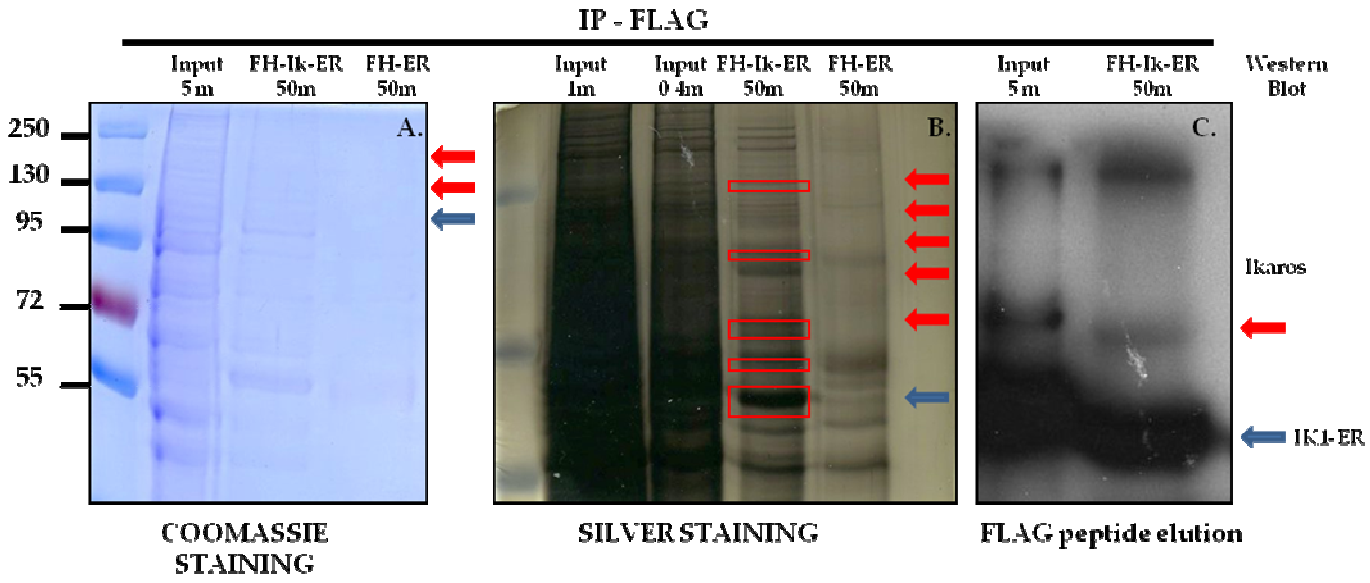
III.10 Detection of the modified Ikaros fractions by Mass Spectrometry.

The presence of several lysines, potential targets for non-canonical sumoylation of Ikaros led us to search for a different approach for revealing where the sumoylation takes place. One such method is the mass spectrometry, which is able to directly map the lysine of SUMO attachment on the target protein. A general description of the steps of this method is listed here:

1. The protein sample digested with protease (e.g. trypsin, chymotrypsin) is loaded onto the MS instrument and undergoes vaporization
2. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles (ions)
3. The ions are separated according to their mass-to-charge ratio in an analyzer by electromagnetic fields
4. The ions are detected by a quantitative method and the signal is processed into mass spectra

Thus, I established an ILC87c cell lines overexpressing the TAP (tandem affinity purification) tagged IK1-ER and the ER part only (as a negative control) both with FLAG-HA tags. In the ER cell line, the estrogen receptor ligand binding domain is expressed as a Simian Virus-40 Nuclear Localization Signal (SV-40 NLS) N-terminal fusion. It is a suitable control for the proteins that bind the ER part of the IK1-ER construct and helps to identify them during the analysis. The need of NLS-ER construct is due to the inefficient nuclear translocation of the ER part when expressed separately from Ikaros upon 4-OHT treatment (data not shown). After doing an IPs with the anti-FLAG antibody, the amount of the immunoprecipitated protein was assessed by two different types of gel stains – Coomassie Blue or Silver staining ($AgNO_3$) (Figure 33). Following the IP, the amount of the modified Ikaros fractions is barely visible on the coomassie gel (Figure 33-A), while loading the same protein amount gives more visible modified Ikaros pattern on a silver-stained gel (Figure 33-B). A mass spectrometry was performed on excised bands (red open boxes on Figure 33-B) missing in the negative control lane. With this starting amount of material, only peptides from the first (unmodified Ikaros band) were detected. The analysis of the upper bands could not detect even Ikaros peptides, suggesting that the amount of the immunoprecipitated protein is below the detection limit of this technique. Thus, in order to enrich the protein amount, instead of boiling of the IP beads in sample buffer, the bound proteins were eluted by an excess

of FLAG peptide and concentrated by Amicon tubes (Figure 33-C). The western blot reveals that the elution of the protein from the beads is successful but the amount of the recovered modified Ikaros in the sample is not satisfactory, showing the limitations of this method in term of isolation



of high amount of posttranslationally modified proteins.

Figure 33. Coomassie Blue and Sivler staining of Immunoprecipitated FLAG-HA-IK1-ER .

(**A**) ILC87c cells overexpressing FLAG-HA-IK1-ER or FLAG-HA-NLS-ER was treated for 24 h with 100 nM 4-OHT and the nuclear fractions were lysed in the presence of de-sumoylation inhibitors NEM and IAA. The nuclear extracts from 50 million cells were subjected to IPs with anti-FLAG antibody bound to protein G beads. The extracts boiled in 2x sample buffer were subjected to SDS-PAGE on 10 % gel stained with coomassie blue (**A**) or silver stained on 6 % gel (**B**).(**C**) Ikaros western blot on nuclear extracts from the FLAG-HA-IK1-ER cell line, eluted from the beads by excess of FLAG peptide and concentrated by Amicon tubes. FH = FLAG-HA tag

In order to circumvent the limitations of the classical IP method, I decided to use a different purification system – nickel affinity chromatography. This method is based on the high affinity of poly-histidine tag to nickel ions immobilized on NTA-agarose beads. The elution of poly-histidine tagged protein from Ni+ NTA-agarose resin is performed by washing with Imidazole. In order to optimize the experimental set up, I used the 293T HEK cell line for ectopic expression of both Ikaros and SUMO-1. I transfected 293T cells with expression plasmids containing untagged Ikaros and 6xHis-tagged SUMO-1. 24h later, a total cell extract was prepared in the presence of NEM and IAA, passed through Ni-NTA-agarose resin, eluted and concentrated by Amicon tubes. A part

of the extract from each step was subjected to SDS-PAGE and western blot with Ikaros antibody (Figure 34).

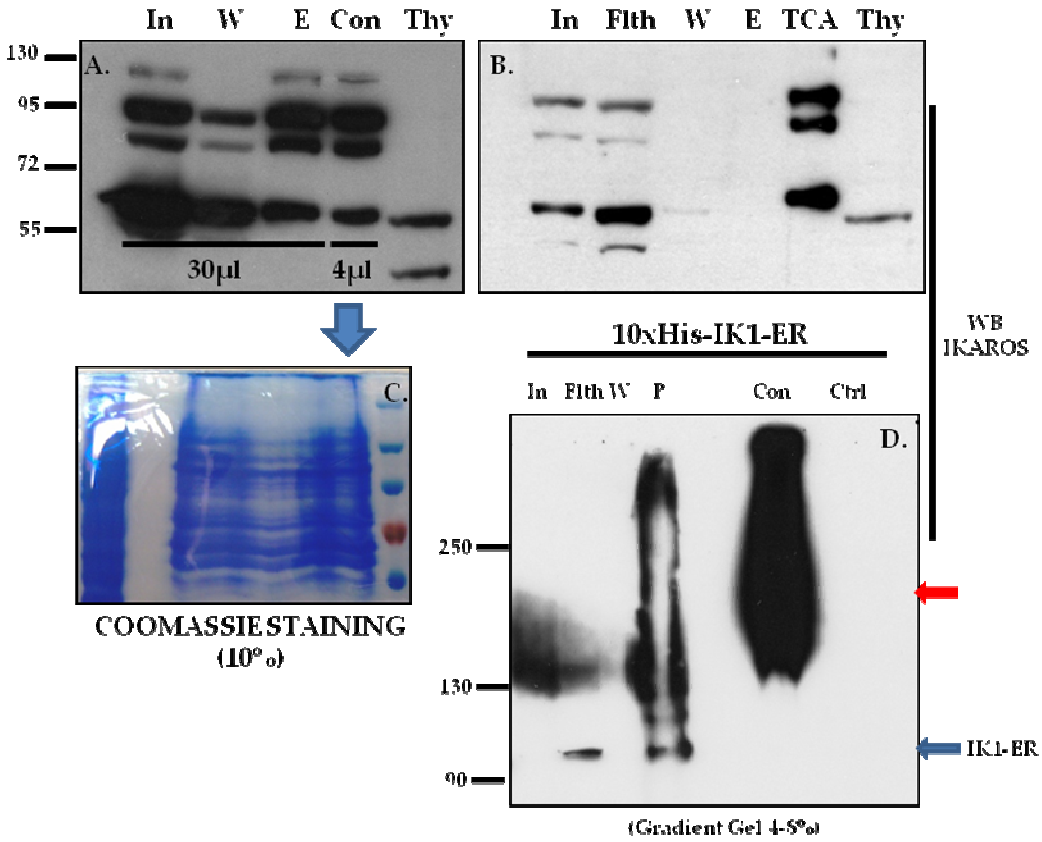


Figure 34. Purification of NTA-Ni+ bound HIS tagged proteins expressed in 293T or ILC87c cells.

(**A**) 293T cells were co-transfected with plasmids containing untagged Ikaros and 6xHis-tagged SUMO-1. 24h later, a total cell extract was prepared in the presence of de-sumoylation inhibitors NEM and IAA and passed through Ni-NTA-agarose resin. The bound proteins were washed, eluted and concentrated by Amicon tubes. The different steps of the purification process were controlled by subjecting an extracts from each step to SDS-PAGE (10% gel) and western blot with anti-Ikaros antibody. The volume of the loaded extract from each step is annotated (**B)** One time freeze-thawed extract from (**A**) was subjected to the same purification steps but the concentration was performed by precipitation with Tri-choloroacetic acid. (**C**) The whole amount of the concentrated protein (the lane "Con" on the western blot from (**A**)) was loaded on 10% SDS-PAGE gel and stained with coomassie blue. (**D**) A nuclear pellet equal to 400 million cells from 10xHis-tagged IK1-ER overexpressing ILC87c cell line was lysed in the presence of NEM and IAA and purified following the same steps as in (**A**).

Legend: In = input, W = wash, E = elution, Con = Concentrate, Thy = total extract from wild type thymocytes used as a positive control, Flth = flow-trough, TCA = Trichloroacetic acid, P = precipitated proteins, Ctrl = concentrated extract from equal number of ILC87c cells.

As we can see on Figure 34-A, the sumoylated Ikaros fractions were successfully isolated end enriched by this approach. In the lane noted as “Con”, 4 micro liters of concentrated protein extract were loaded, resulting in the same amount of detected protein as in the previous steps, suggesting a tenfold concentration. The low amount of unmodified Ikaros that co-elutes with the His-tagged sumoylated fractions is most probably due to the ability of these two fractions to dimerize with each other through their C-terminal zinc fingers under native lysis conditions. After loading the whole amount of concentrated extract (500 µl) into five fused pockets of a 10% gel I stained it with coomassie blue (Figure 34-C). The staining revealed a number of contaminating bands that do not correspond to sumoylated Ikaros fractions. It seems that despite Ikaros and His-SUMO-1 co-expression, the endogenous 293T proteins are also modified to a high extend. The same extract was used for a second round of purification (Figure 34-B), but this time the concentration step was performed by trichloroacetic acid precipitation, resulting in approximately the same degree of enrichment compared to Amicon tubes. Thus, the NTA-Ni+ purification seems to be the method of choice for the enrichment of the modified Ikaros fractions. Therefore, I cloned IK1-ER as N-terminal 10xHis tag fusion and established a new ILC87c cell line. The 10xHis tag has a higher affinity to nickel ions, allowing washing steps with higher Imidazole concentrations, leading to more pure eluted fractions (Figure 34-D). The nuclear pellet equal to 400 million cells from 10xHis-tagged IK1-ER overexpressing ILC87c cell line was lysed in the presence of NEM and IAA and purified following the same steps as in figure 34-A. In order to load the whole amount of the eluted extract in just one well of the gel, I concentrated the sample to a final volume of 40 micro liters. This overconcentration led to a higher viscosity of the extract and partial precipitation – lane “P” (Figure 34-D) in which some amount of IK1-ER was lost. Additional problem of the higher viscosity of the sample is the altered pattern of migration on the gel. As it can be seen from Figure 34-D, modified and unmodified IK1-ER migrate together, resulting in a smear-like pattern. In this case, the limitations of the overconcentration of the sample can be overcome by precipitation of the eluted extract by TCA as shown on Figure 34-B. The obtained results of this part of my work show that the amount of the modified Ikaros fractions obtained after an ordinary IP assays is too low to be subjected to a mass spectrometry analysis. I established an efficient system to purify the sumoylated Ikaros fractions from T-cell line, but the protocol still needs further optimization.

III.11 Sumoylation status of Ikaros family members overexpressed as ER fusions in IK -/- T-cell line

Taking into account that Ikaros is a heavily sumoylated protein, we decided to check whether the other members of the Ikaros family can be targets of sumoylation in the same model system. Inspection of Helios, Aiolos, Eos and Pegasus protein sequence shows that they also contain consensus or potential non-consensus sumoylation sites and a SUMO interacting motif (SIM) (Figure 37). I cloned Helios, Aiolos and Eos in a retrovirus as ER fusions and overexpressed them in the Ikaros -/- ILC87c cell line. Their expression was normalized by sorting the cells with the same GFP intensity (data not shown). To check whether they undergo nuclear translocation similarly to Ikaros-ER, the cells were treated either with ethanol or 4-OHT and the amount of the proteins in the cytosol and nucleus was tested by western blot with the anti-ER antibody. Unexpectedly, upon 4-OHT treatment, for reasons that have yet to be identified, the nuclear translocation of Aiolos-ER is not efficient compared to the other family members, while Eos-ER shows nuclear localization even in the ethanol treated control (Figure 35).

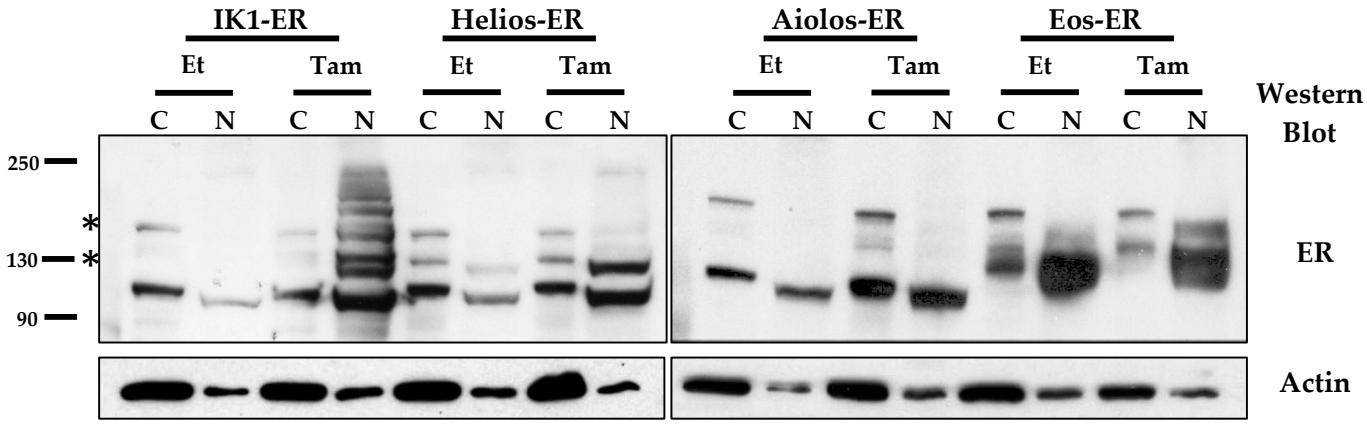


Figure 35. Overexpression of Ikaros family members as ER fusions in the ILC87c cell line.

ILC87c cells overexpressing IK1-ER, Helios-ER, Aiolos-ER and Eos-ER were treated for 24 h with 100 nM 4-OHT and lysed in the presence of NEM and IAA. Cytosolic and nuclear fractions equal to 10 million cells were subjected to SDS-PAGE on 10% gel and tested with anti ER antibody. The beta actin is shown as a loading control. The unspecific bands recognized by the antibody in the cytosolic fractions are labeled with asterisks.

In the case of Eos, a possible explanation might be the existence of a potent nuclear localization signal (NLS) that efficiently transfers the protein to the nucleus in the absence of 4-OHT

treatment. Despite this drawback I checked whether the other family members are modified posttranslationally. Upon 4-OHT treatment, equal amounts of nuclear pellets of each cell line were lysed in 0.5% NP-40 containing buffer supplemented with NEM and IAA, proteins were immunoprecipitated with anti-ER antibody and separated by SDS-PAGE on 4 - 20 % NUPAGE gradient gels. The membranes were probed with anti-ER, anti-SUMO-1 and -SUMO-2/3 as well as anti-ubiquitin antibodies (Figure 36). Compared to Ikaros, the other family members are also posttranslationally modified, although to a lesser extent. All of them show a positive signal for the SUMO isoforms and are negative when probed for ubiquitin. The amount of modified IK1-ER is higher compared to the other family members, resulting in very strong, saturating signal, whereas a short exposure time (see “short exposure” SUMO-1 western blot) reveals its typical modified pattern. Helios-ER shows a similar pattern of migration of the modified fractions, while Eos (at this exposure time) shows just one modified band positive for both SUMO-1 and SUMO-2/3, suggesting the presence of a mixture of mono-modified by SUMO-1 and SUMO-2/3 fractions. Despite the fact that the anti-ER antibody detects modified Aiolos-ER fractions, its inefficient nuclear translocation upon 4-OHT treatment results in very weak signal of the modified fractions that makes the estimation of its modification profile difficult. As discussed above, the posttranslational modification may be cell or cell-stage dependent process. I also have to note that the most of the lysine residues, which are modified in Ikaros are not conserved in Aiolos.

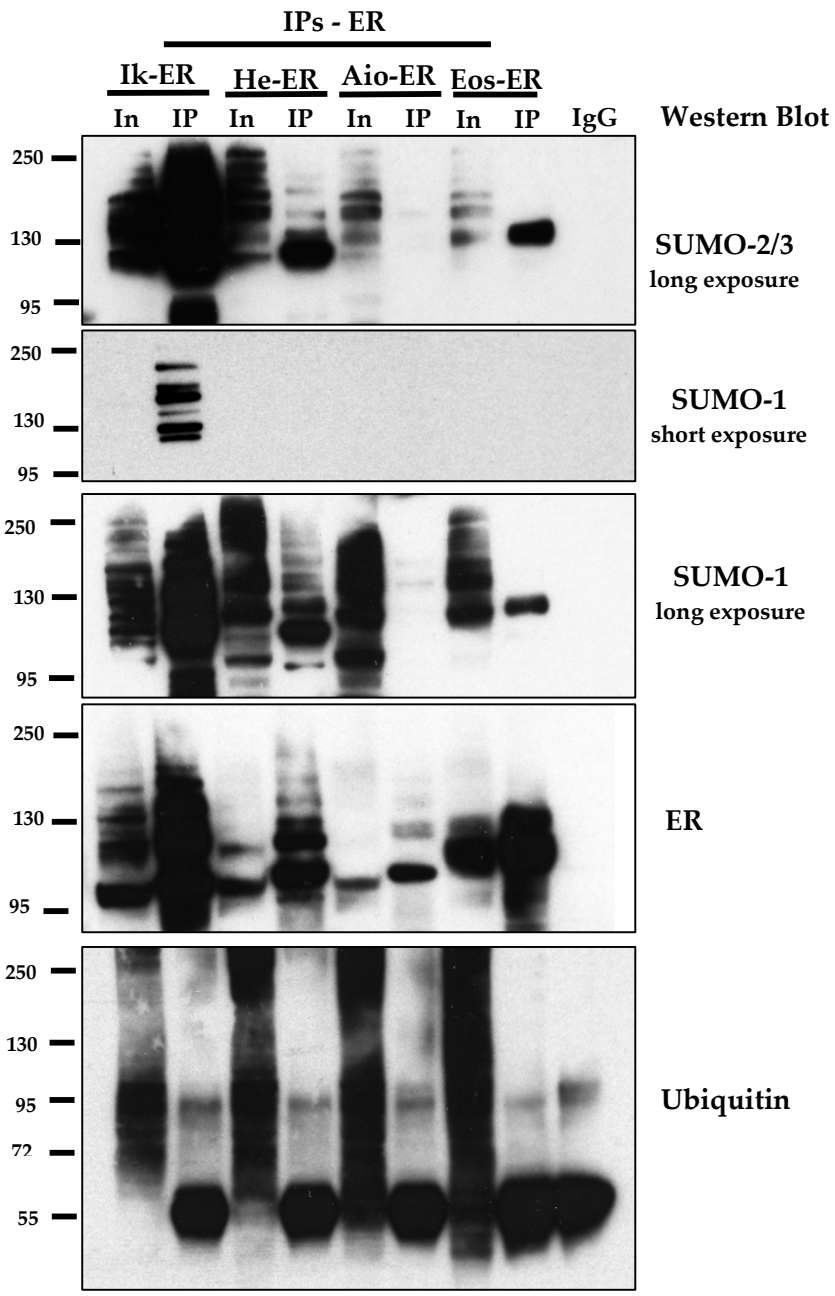


Figure 36. Ikaros family members are modified by SUMO-1 and SUMO-2/3 but not Ubiquitin in Ikaros null T- cell line.

ILC87c cells overexpressing Ikaros, Helios, Aiolos and Eos as estrogen receptor ligand binding domain fusions (ER) were treated for 24 h with 100 nM 4-OHT and 20 uM MG132 2 hours prior the lysis then lysed in buffer containing 0.5 % NP-40 in the presence of de-sumoylation inhibitors - 10 mM NEM and 10 mM IAA. The nuclear extracts from 50 million cells were subjected to IPs with anti-ER antibody bound to protein G beads. The extracts were subjected to PAGE on a gradient gels NUPAGE (4-20%), transferred to PVDF membrane and blotted with the indicated antibodies. In = Input (5%); IP = Immunoprecipitation; IgG = mouse IgG used as negative control for the IP on IK-ER nuclear extract.

Figure 37. Ikaros family contains Sumo Interacting Motif (SIM), consensus and potential non-consensus sumoylation sites.

Ikaros family of proteins contains consensus (black line open bars) and potential non-consensus (red line open bars) sumoylation sites (inverted or ending with amino acid residue that can be phosphorylated).

III.12 Functional characterization of the role of Ikaros sumoylation

After uncovering two additional sumo acceptor sites on Ikaros (K118 and K425) by biochemical approaches, we wanted to determine the role of Ikaros sumoylation in its function, using functional assays. Ikaros is a transcriptional repressor which leads to inhibition of cell proliferation and cell death, when overexpressed in T-cell lines. Thus, comparing the anti-proliferation (AP) ability of Ikaros and its sumo-deficient mutants would give a clue to the function of Ikaros sumoylation. We set up a competition assay in which the AP abilities of IK1-ER and its mutants can be measured by flow cytometry. This is possible because in each T-cell line the expression of the protein (WT or mutant) is coupled with GFP by an IRES and normalized by sorting cells with the same GFP intensity. Therefore, measuring the percentage of the GFP positive cells (GFP+) and their numbers daily, upon six days of 4-OHT treatment would show us differences in the functional properties of the mutants. The principle of the competition assay is shown on figure 38.

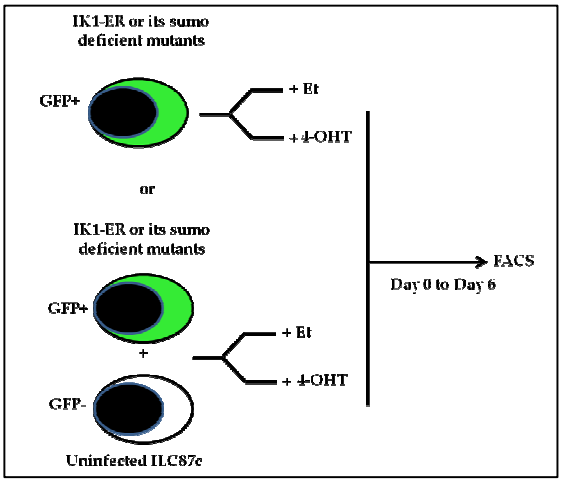


Figure 38. The competition between IK1-ER or its sumo-deficient mutants with the ILC87c (uninfected) cell line.

IK1-ER and its sumo-deficient or deletion mutants (alone or as a mixture with ILC87c) were treated with ethanol or 4-OHT for 6 days. The percentage of the living GFP+ cells was determined daily by flow cytometry. The addition of equal numbers of uninfected ILC87c (GFP-) cells would increase the selective pressure on the tested cell lines making any difference in the proliferation more obvious compared to the controls samples (the pure cell lines).

This approach was chosen because without a competitor cell line, the 4-OHT treatment of IK1-ER and its mutants doesn't have any dramatic effect on the cell proliferation, compared to the ethanol treated samples. Significant differences in their functions appear only in the presence of competitor cells. Despite the obvious absence of SUMO-loss, the K15R and K171R single point

mutants, these cell lines were also tested along with the sumo-deficient mutants – DMZF, TM and 5KR, because of their unusually high proliferation rate, suggesting the existence of modification other than sumoylation. It is known that the lysine residues of non-histone proteins can be subjected to acetylation and methylation (Huang et al., 2008). The deletion mutants like Δ123, ΔDBD and ΔDOM were also included in this assay as control cell lines. The N-terminal mutant Δ123 which lacks the first three exons was used as a control cell line for the K15R mutant, because the deletion includes this site. Therefore, the mutation of this lysine to arginine should not result in a significant functional change compared to the Δ123 deletion mutant. The ΔDBD mutant cell line is used for the K171R point mutant for the same reason. The deletion mutant ΔDOM is used to assess the absence of both the SUMO interacting motif (SIM) and the main PEST domain (the major phosphorylation domain) on the AP abilities of Ikaros (Figure 39-A).

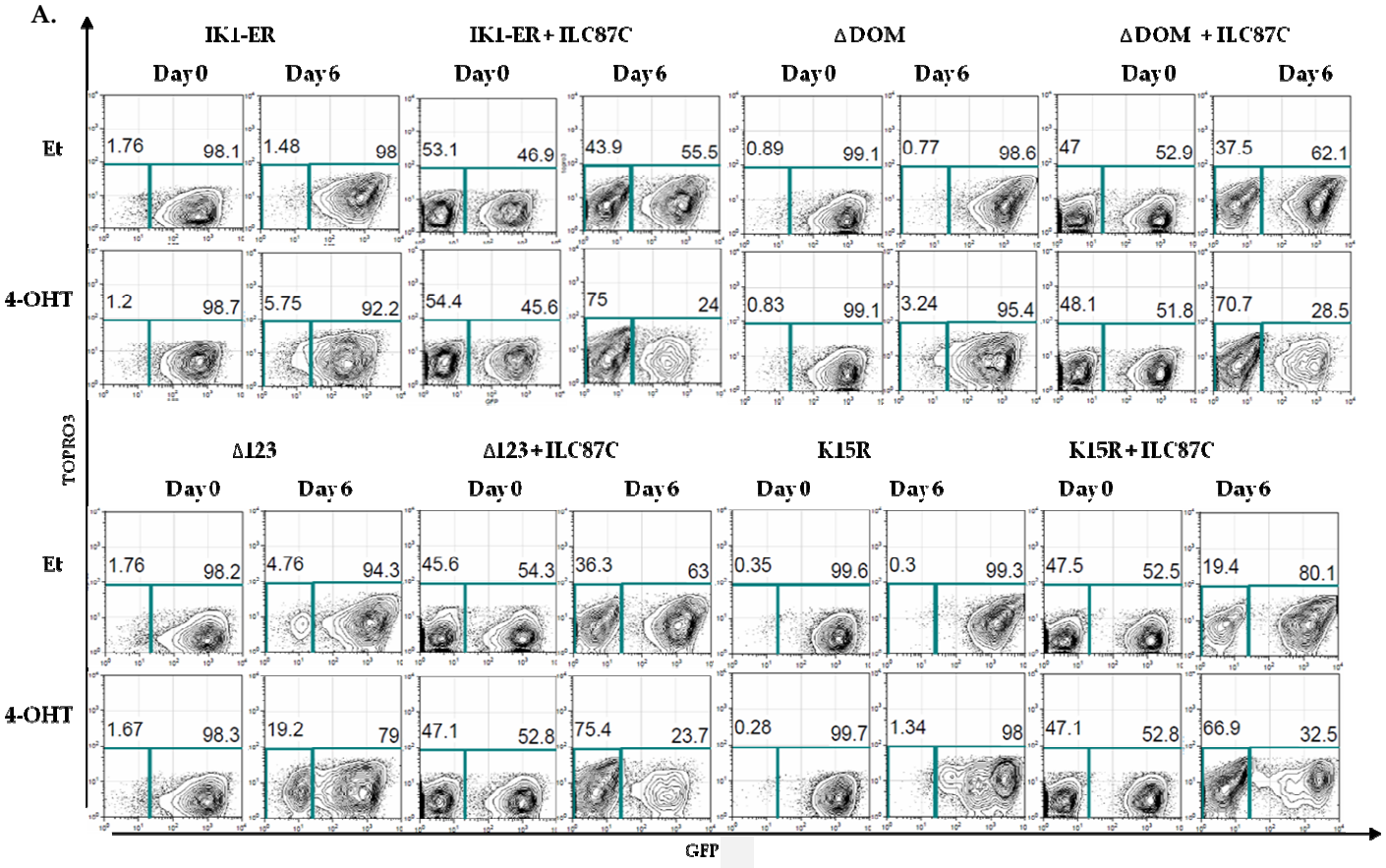


Figure 39. The competition between IK1-ER, ΔDOM, Δ123 and K15R with ILC87c (uninfected) cell line. (**A**) IK1-ER and the indicated mutants (alone or as a mixture with ILC87c) were treated with ethanol or 100 nM 4-OHT for 6 days. The percentage of the living GFP+ cells was determined daily by flow cytometry using TOPRO3 staining. The medium supplemented with Ethanol or 4-OHT was changed daily.

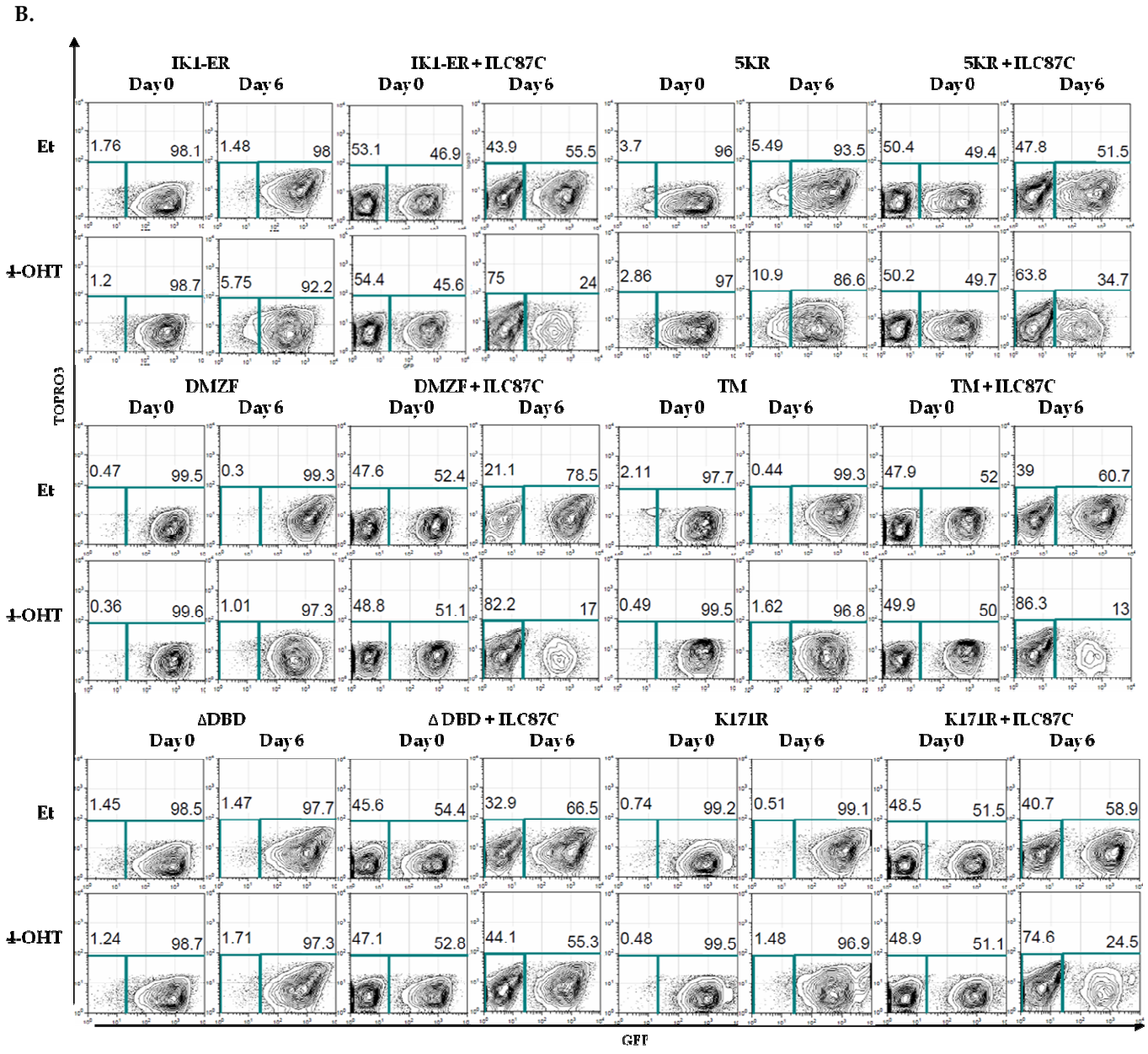


Figure 39. The competition between IK1-ER, DMZF, TM, 5KR, ΔDBD and K171R with ILC87c (uninfected) cell line.

(**B**) IK1-ER and the indicated mutants (alone or as a mixture with ILC87c) were treated with ethanol or 100 nM 4-OHT for 6 days. The percentage of the living GFP+ cells was determined daily by flow cytometry using TOPRO3 staining. The medium supplemented with ethanol or 4-OHT was changed daily.

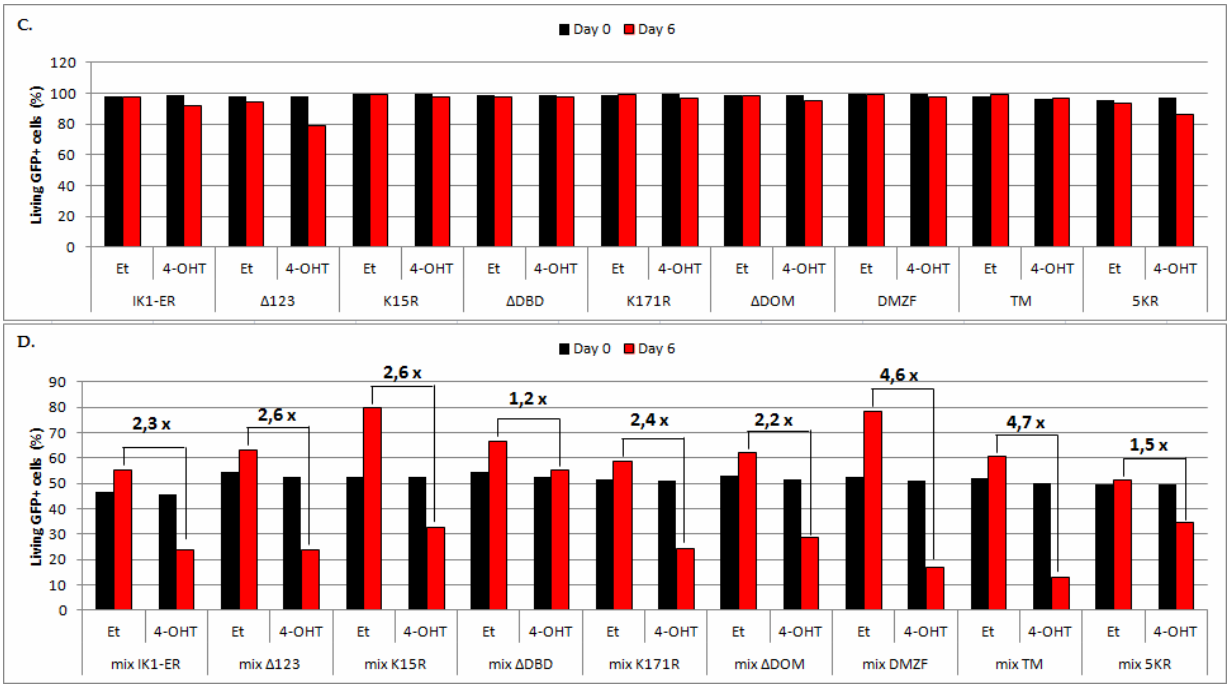


Figure 39 (C, D). Graphical representation of the competition assay between IK1-ER or the indicated mutants with ILC87c (uninfected) cell line.

IK1-ER and the indicated mutants alone (**C**) or as a mixture with ILC87 (**D**) were treated with ethanol or 100 nM 4-OHT for 6 days. The medium supplemented with Ethanol or 4-OHT was changed daily. The percentage of the living GFP+ cells was determined daily by flow cytometry using TOPRO3 staining. The decrease in the percentage of the living GFP+ cells is shown as a fold change (**D**).

In the absence of a competitor cell line, the 4-OHT treatment of IK1-ER and its mutants compared to the ethanol treated controls doesn't have any dramatic effect on the cell proliferation (Figure 39-C). However, their AP function becomes more obvious in the presence of the competitor cells (Figure 19-D). The Δ123, K15R, K171R and ΔDOM mutants show no significant difference in their AP abilities compared to IK1-ER (Figure 39-A, B and D). As expected, the ΔDBD mutant, who lacks the DNA binding domain, showed essentially no AP ability, while the SUMO-deficient mutants DMZF and TM are much more potent compared to IK1-ER with more than fourfold increase of their AP properties. This observation is in agreement with previously published results (Gómez-del Arco et al., 2005) which describe Ikaros sumoylation as a mechanism leading to a decrease of its repressive abilities, impairing its interaction with components of HDAC dependent or HDAC independent repressive complexes. This conclusion leads to the speculation that the less sumoylated is Ikaros, the stronger are its AP functions. However, the DMZF mutant in which only one sumoylated fraction is lost (Figure 28) shows the same AP properties as the TM mutant, in which all, except two modified bands are lost. In agreement with this observation, the 5KR

mutant should possess the strongest AP abilities, because of the almost complete absence of sumoylation. Unexpectedly, the 5KR mutant AP ability is close to the one of the ΔDBD mutant, suggesting more complex role of the sumoylation than a simple physical impairment of Ikaros-partners interactions.

Interesingly, despite its normal AP abilities, the K15R mutant, in which the sumoylation is not impaired, shows an interesting phenotype (Figure 39-A). In presence or absence of competitors, upon six days of 4-OHT treatment this mutant not only maintains but even increases its expression, leading to the selection of a new GFP^{high} population, absent at the beginning of the culture. At day 6 of an extended 9 days culture, this mutant starts to outcompete the competitor cell line with a rate of 10 % per 24 hours (data not shown), suggesting an important function of the K15 in Ikaros function. In fact, if we compare the GFP negative population of this mutant with the same population of the Δ123 mutant at day 6 in the absence of competitors, upon 4-OHT treatment (Figure 39-A), we can see a significant difference. The Δ123 mutant shows 19.2 % of GFP- cells against 1.34 % for K15R, suggesting that the loss of the first three exons confers stronger AP abilities than K15R mutation only. This result suggests that K15 might be modified by other types of posttranslational modifications, such as acetylation or methylation.

III.13 Studying the repressive abilities of Ikaros and its mutants in reporter gene expression experiments.

The strong gain of AP phenotype in the DMZF in which only one sumo site is lost, the significant loss of repression in the 5KR mutant, in which four sumo fractions are lost and the fact that the NURD component MTA-2 co-immunoprecipitates with all of the tested mutants suggest a complex role for Ikaros sumoylation in regulating its functions. To directly assess the repressive functions of Ikaros and its mutants, I cloned the K15R, DMZF, TM and 5KR mutants without the ER part in expression vectors which were used in luciferase reporter gene assay. In this experiment, Ikaros and its mutants are co-transfected in equal amounts along with two additional plasmids: Hes1-Luc and NIC1. The Hes1-Luc plasmid contains the coding sequence of the firefly luciferase gene, an enzyme, which oxidizes its substrate luciferin, leading to bioluminescent emission.

$$\text{luciferin} + \text{ATP} \xrightarrow{\text{Luciferase}} \text{luciferyl adenylate} + PP_i$$

$$\text{luciferyl adenylate} + O_2 \rightarrow \text{oxyluciferin} + \text{AMP} + \text{light}$$

The luciferase gene expression is under the control of an Ikaros target gene-Hes1. Its promotor contains two Ikaros consensus binding sites –TGGGAA. Hes1 gene promoter is directly bound by Ikaros in primary T-cells and T-cell lines, leading to the transcriptional repression of this gene (Kleinmann et al., 2008). On the other hand, the transcriptional activation of Hes1 gene is dependent on the intracellular part of the cleaved NOTCH1 receptor (NIC1), used in this assay as a transcriptional activator of the luciferase expression. Thus, co-transfection of HeLa cells with Hes1-Luc and NIC1 leads to the stimulation of the luciferase expression, while addition of Ikaros represses the transcription and reduces the levels of this enzyme. In this experiment the luciferase expression was normalized to Beta-galactosidase activity, by transfecting equal amounts of B-gal plasmid along with the tested mutants (Figure 40).

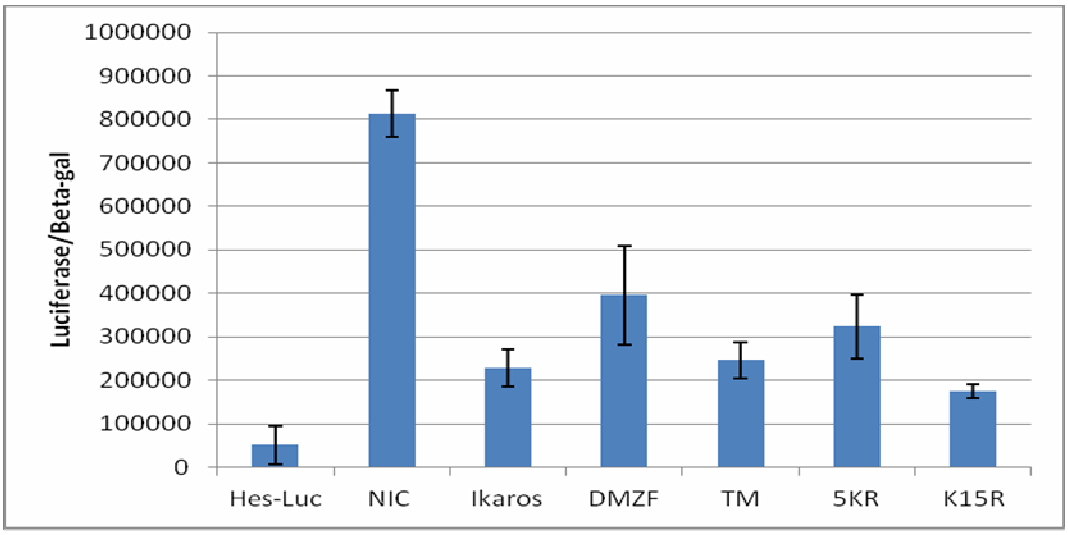


Figure 40. Luciferase reporter assay estimating the repressive abilities of Ikaros and its mutants in HeLa cells.

HeLa cells were transfected with 300 ng of each of the indicated constructs, along with Beta-gal plasmid (100 ng) used as a control to which the luciferase expression was normalized. The lanes annotated as Ikaros, DMZF, TM, 5KR and K15R contain as well the plasmids Hes1-Luc and NIC1. 24 hours post transfection the cells were lysed and the cell extracts were subjected for Luciferase assay. The results of two different experiments performed in triplicates are shown.

The transfection of the Hes1-Luc plasmid alone does not lead to significant expression of the luciferase gene, while the addition of NIC1 increased the luciferase activity up to sixteen times. As expected, Ikaros expression reduced the luciferase activity four times. The different Ikaros mutants showed different levels of repressive abilities with no dramatic change compared to WT Ikaros. Interestingly, in HeLa cells the DMZF mutant is a weaker repressor compared to a T-cell line in which the same mutant exerts strong repression on the cell proliferation (Figure 39-D). This result questions one more time the relevance of the ectopic gene expression experiments in studying the transcription factor function.

III.14 Transcriptome analysis of Ikaros and its point mutants

The data obtained by the reporter gene expression assay doesn't match the more relevant situation in T-cell line, which led us to use microarrays as more reliable source of information about the AP functions of Ikaros and its mutants. The mRNA of each cell line was extracted and subjected to transcriptome analysis in two different experiments on ethanol or 4-OHT 24 hours treated cell lines -IK1-ER, K15R, DMZF and TM.

If we assume that the posttranslational modifications of Ikaros might modulate its stability or its interaction with NURD components only, we would expect to see a different extend of deregulation of the same target genes in IK1-ER and its mutants. Interestingly, the analysis of the samples revealed no significant bias in the gene expression profiles of the mutants, compared to IK1-ER. The genes were selected for analysis by setting a de-regulation threshold of 0.5 fold up- or down-regulation upon 4-OHT treatment of IK1-ER, compared to the ethanol treated control sample. The transcriptome profiles in two separate experiments were compared. The expression of the genes, which showed 0.5 fold de-regulation (up- or down-) in 4-OHT treated IK1-ER were further compared with the expression of same genes in the mutants upon 4-OHT treatment (Figure 41-A). Only the genes de-regulated more than 1.5 times (up- and down-) or more than 0.5 fold less de-regulated (up- and down-) compared to IK1-ER were selected for further analysis (Figure 41-B,C).

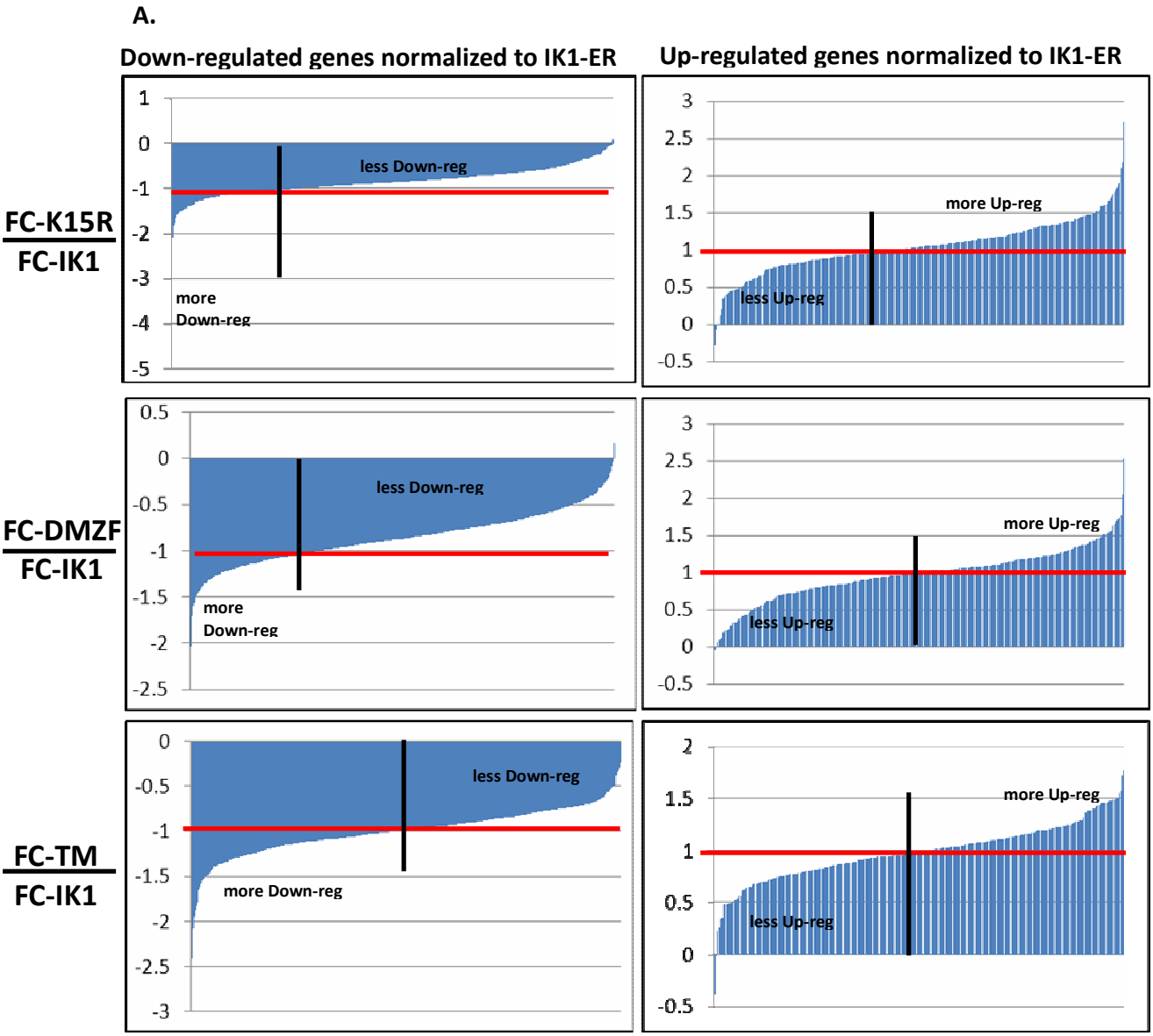


Figure 41. Transcriptome analysis of 4-OHT treated IK1-ER and its point mutants.

(**A**) The first selection step was to select the genes that are Up- or Down- regulated more than 0.5 fold in 4-OHT treated IK1-ER compared to the same cells treated with ethanol. The second step was to normalize the values of these genes (Fold Change - FC) to their value in 4-OHT treated IK1-ER sample. Thus, the red line represents the value of each gene in 4-OHT treated IK1-ER cell line. In case of the down-regulated set of genes (left column), all the genes that are below the red line are more strongly repressed in the indicated mutant, compared to IK1-ER, while all the genes above the red line are less repressed. The same for the genes that are Up-regulated (right column). The black bar represents the crossing point in which the genes are de-regulated to the same extend as in IK1-ER (Value =1).

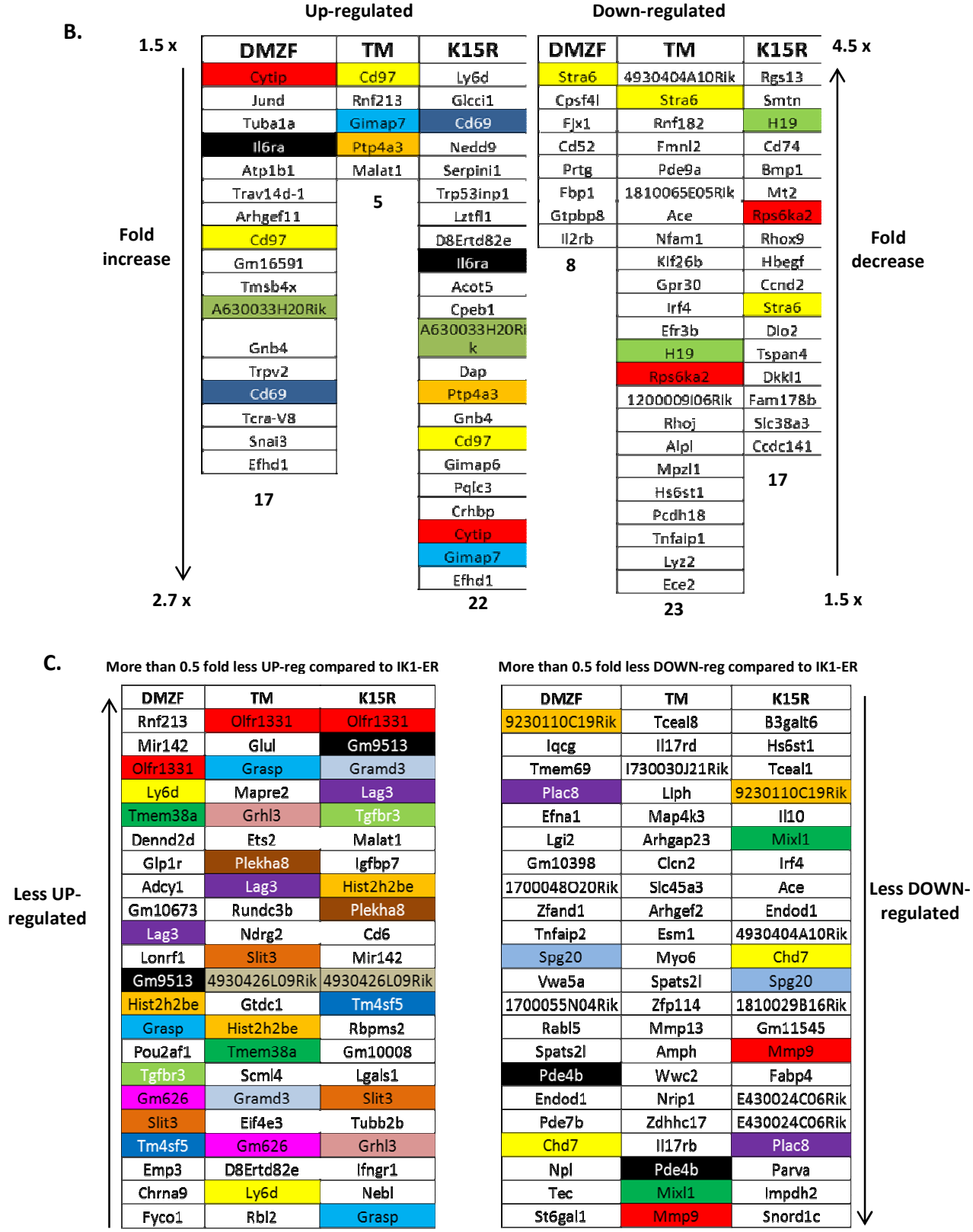


Figure 41. (**B**) Genes de-regulated more than 1.5x compared to IK1-ER + 4-OHT. (**C**) Top 22 genes, 0.5 fold less up- or down- regulated in each of indicated mutants, compared to IK1-ER + 4-OHT. The same color shows the same gene in a different mutant.

The red line in figure 41-A represents the value of a given gene in 4-OHT treated IK1-ER cell line. In case of the down-regulated set of genes (left column), all the genes that are below the red line are more strongly repressed in the indicated mutants, compared to IK1-ER, while all the genes above the red line are less repressed. The opposite is for the genes that are up-regulated (right column). The ones that are below the red line are less up-regulated, while those above the line are more strongly up-regulated. As we can see, the transcriptome analysis of the different mutants shows no striking effect of the sumo deficiency on the Ikaros mediated transcriptional regulation (Figure 41-A). This data doesn't favor the speculation that sumoylation modulates Ikaros stability, because in this case, we would expect to see a stronger general impact on the Ikaros-mediated transcriptional regulation in the mutants. The majority of the genes in the DMZF and TM are up- or down- regulated to a similar extend, compared to IK1-ER. The DMZF shows a bias toward less efficient general repression, because the crossing point is shifted to the left part of the scale. Interestingly, a small subset of genes is strongly repressed by DMZF, suggesting a complex role of the sumoylation on Ikaros-mediated transcriptional regulation. The same situation can be observed for the genes that are up-regulated by the DMZF. Similarly to the DMZF, the TM is also less efficient repressor/activator for some genes but stronger repressor/activator for others. The K15R mutation shows the same less efficient repression main effect with one exception. In this mutant a subset of genes are dramatically repressed compared to IK1-ER, suggesting the existence of posttranslational modification on this lysine. The genes that are up- or down- regulated more than 1.5 fold in the three mutants are shown in figure 41-B. This threshold has been set because of two reasons:

1. To select the more up- and down-regulated genes and to compare their nature among the different mutants using different colors.

2. To compare the number of the genes in each mutant that are up-regulated more than 1.5 fold with the number of genes that are down-regulated to the same extend.

As we can see from figure 41-B, 17 genes are up-regulated more than 1.5 fold versus 8 that are down-regulated in the DMZF, confirming the loss of repression phenotype for this mutant. In the case of the TM, 5 genes are up-regulated, while 23 are down-regulated, suggesting a stronger repressive abilities of this mutant. The K15R mutant shows 22 up- versus 17 down-regulated

genes, which suggests no bias towards more efficient up- or down-regulation of the transcription in this mutant. Unlike the sumo-deficient mutants, a few genes among which *Rgs13* are strongly repressed in this mutant.

In figure 41-C are shown the top 22 genes, more than 0.5 fold less up- or down- regulated in each of indicated mutants, compared to IK1-ER + 4-OHT. Interestingly, the gene *Rnf213*, which is up-regulated in the TM, is less up-regulated compared to IK1-ER in the DMZF.

The colors in this figure show if the same genes can be found as up- or down-regulated in the different mutants. As we can see in figure 41-B and C, the genes, which are up-regulated, show less diversity among the mutants, compared to the genes that are down-regulated. This observation suggests that the sumoylation mainly affects Ikaros repressive properties, rather its ability to act as a transcriptional activator.

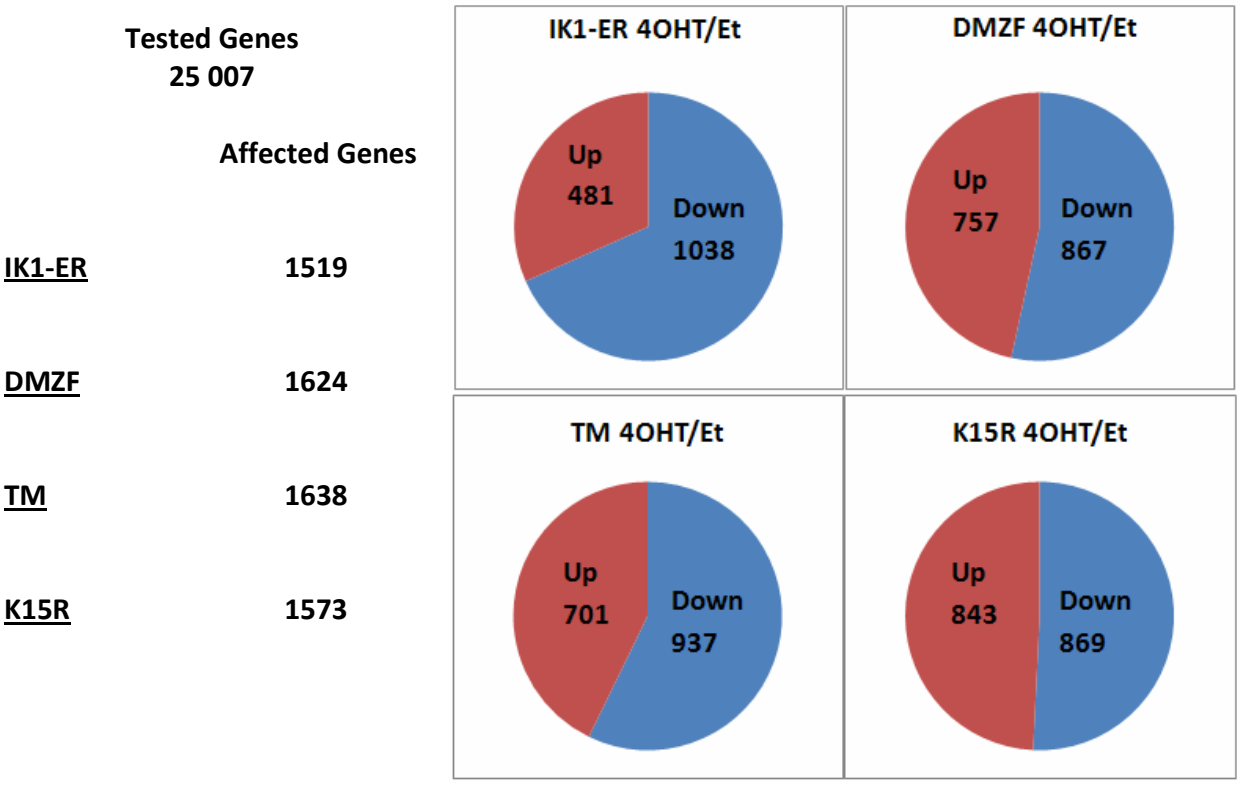


Figure 41. (**D**) The number of the genes de-regulated more than +/- 0.5 fold in 4-OHT treated IK1-ER and its mutants compared to the ethanol treated controls. The red color shows the down-regulated genes, while the yellow color corresponds to the genes, which are up-regulated.

Taken together, all these data favor the idea that sumoylation is a mechanism, which modulates Ikaros-mediated transcriptional regulation. SUMO-deficient mutants affect specific genes and modulate the transcriptional output, without any general pattern. The three lysines 58/240/425 are the major sites of sumoylation which is confirmed by the almost complete SUMO-loss in the TM. The majority of the Ikaros target genes in this mutant were de-regulated similarly to IK1-ER, suggesting that the sumoylation only shapes Ikaros-mediated gene regulation. Interestingly, the K15R mutant shows almost equal number of up- and down-regulated genes, strongly suggesting the presence of posttranslational modification on this lysine (Figure 41-D). The transcriptome data suggests that this posttranslational modification does not directly modulate Ikaros stability, or its intracellular localization. The observation that only a few genes are strongly de-regulated in the mutants and that these genes are different for each mutant, suggests a model in which Ikaros sumoylation can be a promotor dependent process. The mechanisms that underlie the dual Ikaros role - as a transcriptional repressor and activator are less understood. It is assumed that Ikaros itself doesn't have enzymatic activity, which regulates the gene expression, but it interacts and brings to DNA chromatin remodeling complexes able to both repress and activate transcription. In this perspective, the sumoylation of Ikaros can be a mechanism that diversifies the number of its interacting partners, depending on the development stage, promotor context or the extracellular signaling.

CHAPTER 4

Discussion

IV. Ikaros is sumoylated at multiple lysines in primary T-cells and IK null T-cell line

Up to now, the study of Gómez-del Arco and colleagues, 2005 is the only one that addressed the question of Ikaros sumoylation. Their results show that Ikaros is sumoylated in total primary T-cells on two consensus sumoylation sites on Ikaros (K58 and K240), the sumoylation of which leads to loss of Ikaros repressive function in ectopic reporter gene assays. The results of my experiments show that the transcription factor Ikaros is a target of multiple sumoylation events in primary T lymphocytes, as well as in the T-cell line ILC87c. In primary cells, the sumoylation is more obvious during the double negative - DN3 and DN4 stages, suggesting an important functional outcome of this modification in Ikaros functions during the early stages of T-cell development. The study of Gómez-del Arco and colleagues reported the existence of two consensus sumoylation sites on Ikaros, the sumoylation of which disrupts its participation in both histone deacetylase (HDAC) mediated and independent repression. In my experiments, I used ILC87c T-cell line as a model to study the posttranslational modifications of Ikaros. In this experimental model, when overexpressed as an ER fusion, upon 4-OHT treatment, Ikaros shows the same pattern of modified fractions as during the DN4 stage in primary thymocytes. Using antibodies specific for Ikaros, SUMO-1 or SUMO2/3 proteins I detected the presence of seven modified bands that gave positive signal when probed with these antibodies. The detection of the complete modified profile requires the use of two different de-sumoylation inhibitors N-ethylmaleimide and Iodoacetic acid. Only a combination of these strong alkylating agents rescues all the modified fractions during cell lysis. This observation might have two different explanations. The first is that Ikaros, being modified by two different types of SUMO proteins is de-modified by different types of SUMO-specific proteases, the inhibition of which requires two different inhibitors. The second one is that Ikaros is multi-modified just by one type of SUMO, but the de-conjugation of the modifier from different positions requires different de-sumoylation enzymes and therefore is rescued by different inhibitors. The observation that the complete profile of seven sumoylated bands is seen by both SUMO-1 and SUMO2/3 antibodies favors the first option. However, the fact that each of these antibodies recognizes all the seven fractions is controversial. If Ikaros is modified by different types of SUMO proteins, assuming that SUMO-1 and SUMO-2/3

antibodies are not cross-reactive, we should detect different fractions but not all of them when using a given antibody. Thus, it is difficult to conclude if Ikaros is sumoylated by different SUMO proteins or if one of the used SUMO antibodies is cross-reactive.

An interesting point is the size of the modified fractions and their migration pattern on PAGE. When expressed as an ER fusion, Ikaros molecular weight is 100 kD. If we calculate the molecular weight of the mono- and bi-sumoylated bands, simply adding the weight of one or two SUMO proteins (SUMO-1 = 11.56 kDa; SUMO-2 = 10.87 kDa; SUMO-3 = 12.43 kDa), we wouldn't expect to see such a significant separation of the bands in the upper part of the gel. The observation that the fractions 1, 2 and 3 (Figure 31-A, B) are well separated despite being mono-sumoylated is most probably due to the different position of SUMO attachment in each of these fractions. In fact, Pedromo and colleagues showed that the site of modification by SUMO influences the migration of the protein on SDS-PAGE. Thus, the first three modified fractions are mono-sumoylated, while the fractions above them (number 4, 5 and 6) are bi-sumoylated. As expected, the bi-sumoylated fraction 4 is missing in both K58R and K240R single point mutants. The same effect can be seen in K425R mutant, which misses two bands – 3 and 5. The DMZF (K118/K459R) seems to be modified only at position 118 but not at 459, because of two reasons. First, in this mutant only one sumo fraction is lost and second, the single point mutant K459R doesn't show any visible SUMO loss. Interestingly, the loss of sumoylation at position 118 has a dramatic effect on the cell proliferation, comparable to the phenotype of TM (K58/240/K425R), which loses sumoylation on the other three consensus sites in the. The TM shows the presence of only two fractions, while the 5KR mutant doesn't seem to be modified. The extremely low amount of sumoylated fractions in TM and 5KR mutant (seen only after overexposure of the films) makes any conclusion about their number unreliable. The TM shows two bands, one of which is sumoylated at position K118, while the other one is a modification on a non-consensus site. It is difficult to speculate where the modified lysine is, but a recent study identified the estrogen receptor beta as a SUMO-1 target on novel phosphorylated sumoylation motif regulated by glycogen synthase kinase 3beta (GSK3β) (Picard et al., 2012). This motif represents a non-consensus sumoylation sequence I**K**NS, which upon phosphorylation and conversion of the serine in acidic-like residue, mimics the consensus recognition motif I/V/L K x E/D. Thus, the generation of sumoylation motif and its subsequent sumoylation becomes possible only after the conditional phosphorylation of this motif. In case of Ikaros, we can see a similar motif right in front of the C-

terminal zinc fingers K456 – L**K**VY. A tyrosine phosphorylation would convert this motif to a phospho-dependent sumoylation site. In agreement with that, the sumoylation of a number of proteins has been reported to be phosphorylation dependent. The adenoviral oncoprotein E1B-55K, STAT1, Heat Shock Factor 1 (HSF1) and estrogen-related receptor α and γ (ERRs) are sumoylated only after being phosphorylated (Wimmer et al., 2012; Hietakangas et al., 2003; Tremblay et al., 2008).

An interesting observation is that in ILC87c cells, Ikaros sumoylation is fully dependent on its DNA binding ability and partially on its dimerization (Figure 26). Deletion of the DNA binding domain results in complete absence of sumoylation, while the deletion of its dimerization domain dramatically reduces the number of the modified fractions. This result suggests several different explanations. One is that Ikaros is only sumoylated when bound to DNA as a dimer, alone or as a part of a multi-subunit complex. Another possible option is that the deletion of these two crucial regions somehow abolishes the interaction between Ikaros and the modifying enzymes or the protein complex that contains these enzymes. Whatever the reason is, the complete lack of sumoylation in the ΔDBD mutant suggests an important, DNA dependent role for Ikaros sumoylation as a regulator of its functions.

IV.1 The role of posttranslational modifications and Ikaros stability

The stability of a number of transcription factors is regulated by the ubiquitin/proteasome pathway. Unlike the study of Pupescu et al., 2009, which shows a phosphorylation-dependent Ikaros ubiquitination, in my experimental model Ikaros is not a target of ubiquitination. The presence of ubiquitinated fractions can't be detected even after inhibition of the endogenous sumoylation machinery by anacardic acid, suggesting the absence of negative interplay between these two modifications. If positive phospho-ubiquitin interplay exists, this discrepancy might be explained by the different phosphorylation status of Ikaros in the different cell lines used. In their study, Pupescu and colleagues use MOLT-4 - (human T cell leukemia cell line) and VL3-3M2 – (murine thymocyte leukemia cell line) in which Ikaros might be phosphorylated and subsequently

subjected to degradation by the ubiquitin/proteasome pathway. Whether Ikaros is phosphorylated in ILC87c cell line still remains unknown.

An example for SUMO-mediated transcriptional repression by affecting protein stability is forkhead transcription factor Foxl2. In this case, the attachment of SUMO increases its repressive potential by modulating its stability and cellular localization (Marongiu et al., 2010).

Sumoylation-dependent ubiquitination has been reported to regulate the Promyelocytic Leukemia Protein (PML) degradation (Lallemand-Breitenbach et al., 2008; Tatham et al., 2008). Our transcriptome analysis suggests that in our model system Ikaros sumoylation doesn't affect its stability, because of the absence of significant changes in the gene expression profiles of the SUMO-deficient mutants. If the sumoylation affects only the Ikaros stability, we would expect to see a stronger, general de-regulation of the Ikaros target genes in the SUMO-deficient mutants. In order to completely exclude this possibility the stability of IK1-ER and its mutants can be verified by intracellular Ikaros staining of cycloheximide (CXM) treated ILC87c cells. The normalization of the protein expression of Ikaros and its mutants can be achieved by flow cytometry analysis of Ikaros positive stained cells, gated on the same fold expression of GFP. Although we can't exclude the possibility for promoter dependent ubiquitination and degradation of Ikaros, but up to now, there are no data in favor of this kind of regulation.

IV.2 The role of sumoylation in transcriptional regulation

Ikaros is a Kruppel-like DNA binding transcription factor that can both up- or down regulate the expression of its direct or indirect target genes. Ikaros is sumoylated in T cells and my data suggest that this modification is able to modulate its ability to regulate transcription. The sumoylation of one given transcription factor may have a positive or negative effect on the transcription of its target genes. KAP1 (Tif1β) transcriptional repressor is an example for SUMO-mediated transcriptional repression. It contains six sumoylation sites, one of which is at the non-consensus motif QE**K**L. The sumoylation of two of these six positions - K779 and K804 is required for the interaction with chromatin remodelers including nucleosome remodeling deacetylase complex (NuRD), SET domain bifurcated 1 (SETDB1), heterochromatin protein 1 (HP1) and histone

deacetylases (HDACs). These sumo-based interactions lead to KAP1 mediated down-regulation of the transcription and silenced chromatin state (Ivanov et al., 2007; Schultz et al., 2001, 2002; Sripathy et al., 2006; Ryan et al., 1999). The SUMO attachment may also have a dual role on the activity of a given transcription factor. Example for this is the zinc finger transcription factor Sp3, which regulates the expression of spermatocyte-specific and neuronal genes (Stielow et al., 2010). In this study, the authors generated knock-in mouse model that expresses a sumo-deficient mutant form of the Sp3. They found that several spermatocyte and brain-specific genes usually silenced in non-testicular and extra-neuronal tissues of wild-type animals, become aberrantly de-repressed in this mouse. This de-repression is associated with dramatic epigenetic changes, such as DNA methylation decrease and loss of repressive histone methylation marks.

A different way of regulating transcription by sumoylation is to use this modifier as an adaptor protein. Once attached to its target, the sumoylated protein can be specifically recognized and incorporated in a protein complex with chromatin remodeling functions (Garcia-Dominguez et al., 2009) - Table 3. In this case, the target is only recognized by a partner, which contains SUMO interacting motif (SIM).

Complex	Sumo Substrate	Contains SIM	Complex	Sumo Substrate	Contains SIM	Complex	Sumo Substrate	Contains SIM
NURD			**CoREST**			**PCR2**		
Mi-2	√	√	LSD1	√	√	EZH2	√	—
RbAp46	—	—	BHC80	—	—	EED	—	—
RbAp48	—	√	CoREST	√	√	SUZ12	√	—
HDAC1	√	√	HDAC1	√	√	RbAp48/46	—	—
HDAC2	—	—	HDAC2	—	—	**SETDB1**		
MTA1/2	—	√	BRAF35	—	—	SETDB1	—	√
MBD3/2	—	—	ZNF261/X-FIM	—	—	MBD1	√	—
p66	√	—	ZEB1	√	—	MCAF1	√	√
			ZNF198/FIM	√	√	**MEC**		
			ZNF217	—	—	Mi-2	√	√
			CtBP	√	—	dMEP1	—	√
			Pc2	√	—			
			KIAA0182	—	—			
			TFII-I	√	—			

Table 3. Transcription-regulating complexes which include proteins that are sumoylated or containing sumo interacting motif (SIM), adapted from (Dominguez et al., 2009). Legend: (√ = Yes; — = No or unknown).

It seems that sumoylation is crucial for the function of a number of chromatin remodeling complexes. It promotes HDAC-mediated transcriptional repression (Yang et al., 2004), as well as regulates promoter occupancy and gene-specific repression by Polycomb repressive complex 1 (PRC1) (Gill G., 2010). The issues mentioned above are among the examples for SUMO-mediated transcriptional repression, but it does not necessarily mean that sumoylation always negatively regulates transcription. SUMO-dependent transcriptional activation has also been reported. Sumoylation of de novo DNA methyltransferase 3a (Dnmt3a) modulates its interaction with histone deacetylases and its capacity to repress transcription. The attachment of SUMO-1 disrupts the Dnmt3a - HDAC1/2 interaction and thus, its ability to repress transcription in HDAC dependent manner (Ling et al., 2004). Similarly, SUMO-1 attachment mediates inhibition of MBD1 repressive function, disrupting MBD1/SETDB1 complex formation (Lyst et al., 2006). Another example for transcriptional activation is the observation that sumoylation positively regulates beta-catenin dependent transcriptional activation by transcription factor Tcf-4 (Yamamoto et al., 2003) and p53-mediated transcriptional activation (Gostissa et al., 1999; Rodriguez et al., 1999). An interesting observation is the presence of consensus sumoylation sites on the members of the SWI/SNF chromatin remodeling complex, suggesting a possible role of this modification in its function.

All these examples contribute to the better interpretation of our microarray experiment data. Among the genes that are up- or down regulated by IK1-ER and its mutants we can find a subset of genes that are de-regulated at the same level. A subset of genes shows significant difference (up- or down-) between WT protein and its mutants, as well as between the mutants themselves. This result favors a model in which Ikaros sumoylation does not directly modulate its stability, because we would expect to see just a stronger de-regulation effect on the gene expression profile. A similar effect would be seen if the sumoylation was a mechanism that only mechanistically interrupts the interaction of Ikaros with components of the NURD complex. Although both Ikaros and NURD components, such as Mi-2 and HDAC-1 contain SIMs and are targets of sumoylation, my experiments don't suggest that sumoylation is indispensable to Ikaros-NURD complex assembly. The significant difference in the expression profiles of DMZF and TM and the development stage-dependent nature of Ikaros sumoylation in primary T-cells, suggest more complex way of regulation. One possible explanation might be that Ikaros interacts with many, yet unidentified partners in a stage and promoter dependent manner. Unlike its

constitutive interacting partners, the experimental detection of the SUMO-dependent Ikaros partners would require the use of de-sumoylation inhibitors. In theory, we can have a situation, in which Ikaros being bound to its target promoters is differentially sumoylated and thus differentially regulated by the contextual presence or activity of the sumoylation and de-sumoylation enzymes, as well as sumoylated partners. A dynamic sumoylation may occur at specific lysine or as a combination of lysines, recruiting a different set of interaction partners dependent on the promoter or the development stage of the cell. This model is able to explain the simultaneous existence of genes that are unaffected or differentially de-regulated among the mutants. It offers many options for regulation in term of its functional plasticity. For example, it doesn't require the complete assembly or disassembly of the entire chromatin remodeling complex, saving time and energy and doesn't define it as ultimate repressor or activator of the transcription. The promotor dependent recruitment of sumoylated units with activating or repressive activities would result in a differential control of the gene expression. The existence of this functional model can be verified by an appropriate experiment such as chromatin immunoprecipitation sequencing (Chip Seq) by two different antibodies, anti-Ikaros and anti SUMO-1 or SUMO-2/3. This assay would allow mapping Ikaros binding sites on the genomic DNA upon sumoylation. Performing a two-step Chip experiment with these antibodies and subsequent sequencing of the DNA would reveal the genomic fragments bound by sumoylated Ikaros. Another approach is the use of shRNA mediated SUMO knock-down and one step Ikaros Chip Seq experiment. In this case, the DNA binding profile of Ikaros should be compared with its profile in a control cell line expressing endogenous SUMO proteins.

Reporter gene expression experiments show no significant difference in the action of Ikaros and its SUMO-deficient mutants as transcriptional repressors. When overexpressed in HeLa cells all the constructs are able to decrease the expression of the luciferase reporter to a different extent. Only the DMZF mutant shows a partial loss of repressive abilities compared to IK1-ER. One of the possible explanations is the inability of this mutant to interact and tether co-repressors to the promoter of the reporter gene. However, in ILC87c cells DMZF interacts with NURD components, therefore the loss of sumoylation at position 118 most probably interferes with the HDAC-independent transcriptional repression of Ikaros. Another possible explanation of this phenotype is a hypothetical change of the phosphorylation status of this mutant, if we accept that interplay between these two posttranslational modifications is possible. A number of additional factors

should be taken into account when studying the role of the posttranslational modifications in the regulation of transcription, such as cell type and development stage. In fact, it has been already reported that the sumoylation of the progesterone receptor modulates its transcriptional activity in a promoter-dependent context (Abdel-Hafiz et al., 2012). Thus, drawing a general conclusion for the role of the sumoylation as a factor modulating transcriptional activity in ectopic gene expression assays seems to be unreliable.

The K15R mutation is interesting issue because it shows an unusual phenotype in the competition and transcriptome assays. During competition, the cells expressing this mutant acquire unusually high GFP expression, without significant reduction of its AP strength. However, in extended nine-day long competition experiment at day six, this mutant loses its AP abilities and quickly outcompetes the ILC87c competitor cell line with rate 10 % per 24 hours (data not shown). Ikaros lysine 15 is clearly not sumoylated but might be a target of a different kind of modification typical for lysine residues, such as acetylation or methylation. There are three observations in favor of this speculation. First, the transcriptome profile of this mutant shows more dramatic de-regulation of some of the same genes that are de-regulated in the sumo-deficient mutants and second, this lysine is surrounded by three residues that are shown to be phosphorylated, strongly suggesting interplay between Ikaros phosphorylation at positions S13, S21 and T23 and this hypothetical modification. Finally, if this lysine is not modified we would not expect to see a difference in the GFP positive cell populations of K15R and Δ123 deletion mutant (which contains this lysine) during the competition assay. Less likely, the replacement of lysine 15 with an arginine may cause this severe phenotype by simple misfolding of the protein. In order to test this possibility a different type of mutation, such as K to Q (lysine to glutamine) should be introduced at the same position. This conversion is used to mimic a constitutively acetylated lysine residue in studies that reveal the Bcl-6 acetylation as a way to inactivate its repressive functions (Fujita et al., 2004; Bereshchenko et al., 2002). If we assume that K15 is for example acetylated, a mutation K to R would result in constant loss of function, while mutation K to Q would result in a constant gain of function. Thus, if the transcriptome profiles of these two mutants are comparable, it would be more reasonable to assume that this phenotype is due to misfolding, rather than to a posttranslational modification. Different transcriptome profiles would suggest that K15 is posttranslationally modified, an event that might increase the number of Ikaros interacting partners or to diversify the pathways that regulate its function.

In fact, the acetylation of zinc finger transcription factors has been shown to be a mechanism which regulates their repressive and activating functions. Transcription factor EKLF (KLF-1) can be both acetylated and sumoylated, as the first modification promotes co-activator recruitment and formation of an open chromatin structure (Sengupta et al., 2008), while the sumoylation is crucial for its repressive abilities, by recruiting Mi-2 NURD component in a SUMO dependent manner (Siatecka et al., 2007). The abilities of another Kruppel-like family member, KLF8, to activate transcription are also regulated by these two types of modifications, as interesting negative interplay between them has been observed (Urvalek et al., 2011).

IV.3 A possible interplay between phosphorylation and sumoylation in regulating Ikaros functions

The dramatic inhibitory effect of DMZF mutant on the cell proliferation, its ability to interact with co-repressors, such as HDAC-1 and MTA-2, in a way similar to WT Ikaros and its transcriptome profile, suggests that this effect might be due to a reason other than gain or loss of NURD components. Ikaros phosphorylation has been reported as a posttranslational modification with profound effect on its DNA binding abilities. In fact, the DNA-binding ability of number of proteins, such as nonhistone high-mobility group I protein (HMG-I), the nuclear oncoprotein c-Myb, c-Jun as well as myogenic helix-loop-helix (HLH) proteins is reduced upon phosphorylation (Reeves et al., 1990; Lüscher et al., 1990; Lin et al., 1992; Li et al., 1992). During mitosis, Ikaros is phosphorylated on three highly conserved motifs that link the four N-terminal DNA binding zinc fingers. The replacement of the phosphorylated residues with phosphomimetic substitutions abolishes the DNA-binding ability of Ikaros and its pericentromeric localization (Dovat et al., 2002). The kinase responsible for the mitotic-dependent phosphorylation of these linker motifs is still unknown. These observations, taken together with the sumoylation of lysine 118, lead to the speculation that interplay between Ikaros phosphorylation and its sumoylation might exists. An interesting example for such interplay is the observation that loss of sumoylation of transcription factor STAT1 dramatically decreases its phosphorylation level (Begitt et al., 2011). Ikaros is phosphorylated by several kinases. CKII has a major role, while GSK3, CamKII, PKA and Cdk have a minor contribution (Gomez-del Arco et al., 2004). Interestingly, CKII has never been reported to

interact with Ikaros in co-immunoprecipitation assays. This kinase undergoes sumoylation by SUMO-2, an event that contributes to the cell cycle regulation by SUMO-mediated phosphorylation (Yao et al., 2011). It is possible that this kinase interacts with Ikaros through its SIM only when they are both sumoylated. It would be interesting to perform a co-immunoprecipitation assay in the presence of both de-sumoylation inhibitors - NEM and IAA and to check by western blot if CKII interacts with Ikaros under these conditions. Up to now, it is assumed that CKII is not implicated in the phosphorylation of the conserved linker motifs because it can't be abolished by treatment with CKII specific inhibitors (Li et al., 2012). Thus, phosphorylation-mediated inhibition of the DNA-binding of Ikaros might be due to another sumoylated kinase that interacts with Ikaros only during mitosis. In fact, protein kinases, such as ERK5 and Aurora-B, protein tyrosine phosphatase 1B (PTP1B) have been reported to be regulated by sumoylation (Woo et al., 2008; Ban et al., 2011; Dadke et al., 2007). However, the interplay between sumoylation and phosphorylation can be dual, as the phosphorylation of several substrates negatively affects their sumoylation. Phosphorylation of IκBα, PML, Elk-1 and c-jun results in reduced sumoylation (Desterro et al., 1998; Everett et al., 1999; Yang et al., 2003; Muller et al., 2000). Interestingly, Ikaros2 (IK2) isoform that lacks the first DNA-binding zinc finger, but still binds DNA should not be sumoylated at K118 because the consensus motif LKCD in this isoform is destroyed. There are not enough experimental data revealing the exact function of IK2, isoform, but in addition to the missing exons, the absence of sumoylation might be one additional reason of their functional diversity.

Another possible explanation for the presence of a sumoylation site right in front of Ikaros DNA binding domain is the direct regulation of its accessibility or steric conformation. This speculation is supported by observations that sumoylation inhibits the DNA-binding in other transcription factors, such as Sox2 and heat shock factors 1 and 2 (HSF-1, HSF-2) (Tsuruzoe et al., 2006; Anckar et al., 2006). Taken together, these data suggest a number of possible ways of regulation of Ikaros function by sumoylation.

IV.4 Conclusion

In my work, I show the presence of additional sumoylation sites on the transcription factor Ikaros, a crucial regulator of the development of hematopoietic system. The functional analysis of the sumo-deficient mutants shows a complex role of this modification in regulating Ikaros transcriptional properties. The identification of these new sumoylation sites might contribute to a better understanding of its dual repressive - activating function, as well as to increase the pool of its conditional interacting partners. Moreover, the existence of different sumoylation pattern of the different Ikaros splicing isoforms would complete the knowledge of their functional diversity.

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