T.C.
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SCIENCE AND ENGINEERING

M.Sc. THESIS

STRUCTURE ANALYSIS OF THE HUMAN NOTCH1-ANKYRIN DOMAIN VIA X-RAY CRYSTALLOGRAPHY AND TARGETING NOTCH TRANSCRIPTION COMPLEX ASSEMBLY BY USING POTENTIAL THERAPEUTIC LIGAND INTERACTION

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FOREWORD

There are many people to thank for supporting me up to this point and encouraging me even at every single step of this journey.

First of all, I want to thank my family for trusting me with my decisions, believing in me at every single step that I take in this journey. Their trust, encouragements means the world to me and always will during this long lasting journey of mine through academia.

I want to thank Kurtulus Golcuk, was my supervisor at Fatih University and was a very open-minded person from very beginning to the end about my research suggestions while being supportive of my thesis.

I thank Sven Hennig for giving me an opportunity and accepting me to his lab, being an understanding, supportive and one of the coolest PI's I have ever seen. I will always remember the things I have learned in his lab. I also thank each of Hennig's Lab Members and people at CGC, who were very welcoming, did not hesitate to help me when I needed and were very nice people indeed.

I am very glad for working with Fabian Moertl who supervised me through this project. There are many things to thank him for but, firstly for his time, then for his patience, endless explanations to my non-ending questions and letting me take a part in this project. It was the right place and right people to restart and refocus on being an academic again. I really learned a lot and I believe this process thought me more than it was intended to.

I also thank my Dad, I thank him a lot. He is the most encouraging person, a wise man, best friend of mine. I literally owe my lifelong education journey to him who put up with me, never gave up on me, led, trusted and supported me in every single step now and then...

Thank you.

April, 2017 Elif ERDEM

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

Symbol	Explanation
M	: Molar
m*M*	: Mili Molar
µl	: Microliters

Abbreviation	Explanation
ANK	: Ankyrin Domain
APS	: Ammonium Persulfate
CHES	: N-Cyclohexyl-2-aminoethanesulfonic acid
DTT	: Dithiothreitol
E. coli	: *Escherichia coli*
EDTA	: Ethylenediaminetetraacetic acid
EGF	: Epidermal Growth Factor
GSH	: Glutathione
GST	: Glutathione-S-Transferase
LB	: Lysogenic broth
MES	: 2-(N-morpholino) ethane sulfonic acid
PAGE	: Polyacrylamide gel electrophoresis
PEG	: Polyethylene glycol
PMSF	: Phenylmethanesulfonyl fluoride
SDS	: Sodium dodecyl sulfate
SOC	: Super Optimal broth with Catabolite repression
TAE	: TRIS-Acetate-EDTA Puffer
TB	: Terrific Broth

ÖZET

YÜKSEK LİSANS TEZİ

INSAN NOTCH-1 ANKYRIN DOMAININ X-ISINI KRISTALLOGRAFISI ILE YAPI ANALIZI VE POTANSIYEL TERAPOTIK LIGAND ETKILESIMLERI KULLANARAK NOTCH TRANSKRIPSIYON KOMPLEKSININ OLUSUMUNUN ENGELLEMESININ HEDEFLENMESI

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Genetik ve Biyomühendislik Anabilim Dalı

Danışman : Yrd. Doç. Dr. Kurtuluş GÖLCÜK

Notch-1 proteininin Ankyrin kismi, Notch transkripsiyon kompleksinin olusumu ve hucre icerisindeki notch yolagi icin onemli bir domaindir. Bu calismada N-terminali ve C-terminali kisaltilmis olan Ankyrin protein fragmenti; protein kristallestirilmesi optimizasyonu, protein yapisi ve Notch transkripsiyon kompleksinin olusumunu engelleme potansiyeli olan kimyasal fragmentler ve proteinler arasindaki muhtemel bir etkilesimin X-ray kristalografisi kullanilarak tespiti hedeflenmistir. Calismada Rekombinant protein ekspresyon ve purifikasyonu, afiniti kromatografisi, HPLC, FPLC, SDS-PAGE karakterizasyonu ve X-ray kristalografisi icin gerekli screen'ler ile kokristalizasyon teknikleri kullanilmistir.

Nisan 2017, 60 sayfa.

Anahtar kelimeler: Molekuler Yolaklar, Notch1, Ankyrin Domain, X-ray Kristallografisi, Protein Saflastirma.

SUMMARY

M.Sc. THESIS

STRUCTURE ANALYSIS OF THE HUMAN NOTCH1-ANKYRIN DOMAIN VIA X-RAY CRYSTALLOGRAPHY AND TARGETING NOTCH TRANSCRIPTION COMPLEX ASSEMBLY BY USING POTENTIAL THERAPEUTIC LIGAND INTERACTION

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Department of Genetics and Bioengineering

Supervisor : Asst. Prof. Dr. Kurtuluş GÖLCÜK

Notch-1 Ankyrin domain is important for Notch Transcription Complex assembly as well as Notch intracellular transcription pathway. In this study, N-termini and C-termini shortened Ankyrin protein fragments were used for optimizing protein crystallization conditions, detecting proteins structure and detection of any potential interaction with the putative fragments which may bind to Ankyrin protein and potentially disrupt the assembly of the Notch transcription complex. In this study, recombinant protein expression, protein purification via affinity chromatography, size exclusion chromatography, characterization via SDS-PAGE, spectrophotometry and initial screening, refinement, co-crystallization, X-ray diffraction and Structure analysis were employed.

April 2017, 60 pages.

Keywords: Molecular Signaling, Notch1, Ankyrin Domain, X-ray Crystallography, Protein Purification.

1. INTRODUCTION

1.1. BACKGROUND

The Notch pathway is one of the most known signaling pathways in the cell metabolism. Although it is well known, its mechanism and function are not thoroughly solved. There is a serious necessity for related studies concerning Notch in which it still needs to be comprehensively studied regarding its functional role and mechanism in cell metabolism.

Notch is an important membrane protein that is being activated by extracellular ligands and following its activation, it works canonically or non-canonically depending on the pathway, it needs to activate or be a part of. The Human Notch protein family consists of four different homologs, called Notch1-4. Any information related to it will bring scientists one step closer to figuring out therapeutics which are specifically designed to deactivate or decrease Notch Transcription Complex activity in cancer cells.

Selectivity within drugs is very important for an effective disease treatment. Selectivity is also a very challenging trait to achieve with the proteins similar to Notch, which is very broad so that it is hard to figure out the structure for designing small molecules which will specifically bind to the Notch then prevent the Notch Transcription complex assembly. There are many studies still going on in industry and academia to figure out this problem and find a target that will specifically bind to Notch Transcriptional Complex and prevent it from transcription or disturb the complex. All in all, it is important to gain more insight into the structure of the binding site of Notch 1 in order to supply more insight about the future studies.

1.2. PURPOSE OF THE STUDY

In this study, we aimed to perform fragment optimization of Notch Intracellular Domain and its crystallization to find a well crystallizing, well diffracting fragment suitable for crystal soaking and co-crystallization while shortening the ankyrin domain on C- and N-termini. That, X-ray Crystallography, Structural Biology, and Molecular Biology related techniques were employed.

1.3. LITERATURE REVIEW

1.3.1. Notch Receptors and Notch Signalling

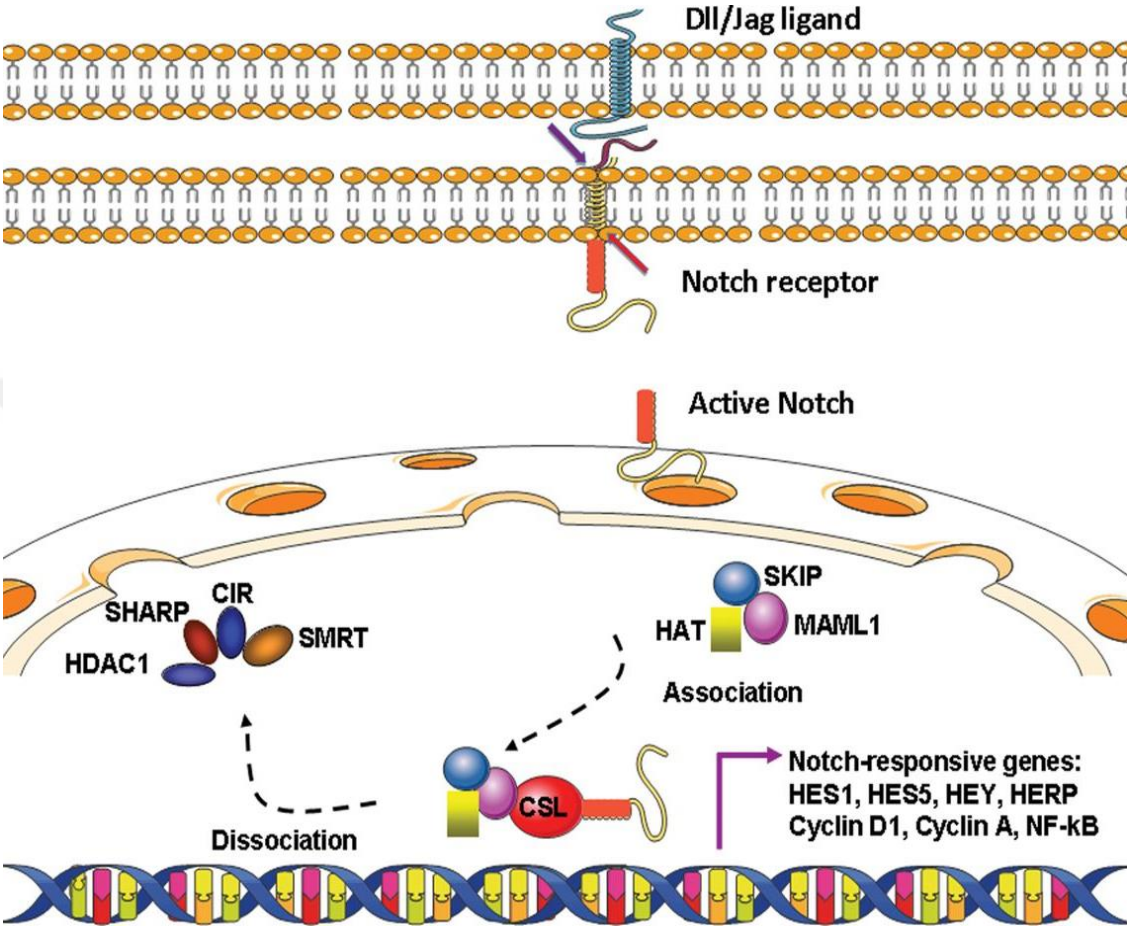


Figure 1.1: Notch signaling. The Notch Pathway(Rizzo, Miele, & Ferrari, 2013).

The first Notch mutant was discovered by Thomas Hunt Morgan in a strain of fruit flies that have "notches" at their wing blades' margin. Notch signaling is known for being important during embryonic, post-natal development and regulates the various cellular processes such as stem cell maintenance, cell death, and cell differentiation. (Borggrefe et al., 2016; Hori, Sen, & Artavanis-Tsakonas, 2013; Koch, Lehal, & Radtke, 2013) A cell-cell contact between the notch and ligand presenting cells is required for the activation of notch signaling pathway. An interaction between the Notch receptor and the ligand, induces the cleavage via catalytic activity of extracellular ADAM metalloprotea (Mumm et al., 2000) and an intracellular Gamma-secretase-containing complex (Fortini,

2002) which then causes the release of the NICD (Notch Intra-Cellular Domain) into the cytoplasm then to the nucleus (Borggrefe et al., 2016).

The Notch receptor is an evolutionarily conserved protein among the species. The Notch family consist of four members which are 60% homologous to each other (Notch1,2,3 and 4) and Drosophila Notch (Callahan & Raafat, 2001; Espinoza & Miele, 2013; Lardelli, Williams, & Lendahl, 1995). It is a single-pass transmembrane protein that consists of three domains which are called as, an extracellular domain (NECD), a transmembrane domain (NTMD) and an intracellular domain (NICD).

The intracellular domain contains an RBP-jk association module (RAM) which interacts RBP-jk (also called as CSL) which acts as a coactivator and induces the assembly of the transcriptional complex (Espinoza & Miele, 2013; Tamura et al., 1995). Following the RAM domain, there is seven ankyrin (ANK) repeats which interacts with CSL as well as other transcriptional regulatory factors (Nam, Sliz, Song, Aster, & Blacklow, 2006), two nuclear localisation signals (NILSs), a transactivation domain(TAD) and C-Terminal PEST sequence, a region that contains many proline, glutamic acid, serine and threonine, is very important for the stability of Notch intracellular domain via its multiple phosphorylation sites. It also triggers the ubiquitination and turnover of the receptor (Espinoza & Miele, 2013; Rechsteiner, 1988).

1.3.2. Notch Transcription Complex, Assembly and Function

Notch receptors play a role in intracellular signaling, which is important for development between adjacent cells via forming a complex to perform a transcription of target genes upon its activation. Since Notch signaling controls various cell-fate specific events, any dysregulation during the signaling causes a number of diseases because of the deficiencies during the development process (Fortini, 2009). The Notch Transcription Complex (henceforth referred as NTC) is a ternary complex formed by a widespread network of protein–protein interactions, including Notch Intracellular Domain (NICD), CSL (CBF1/suppressor of Hairless/Lag-1) and MAML (Mastermind-like1) (Choi et al., 2012).

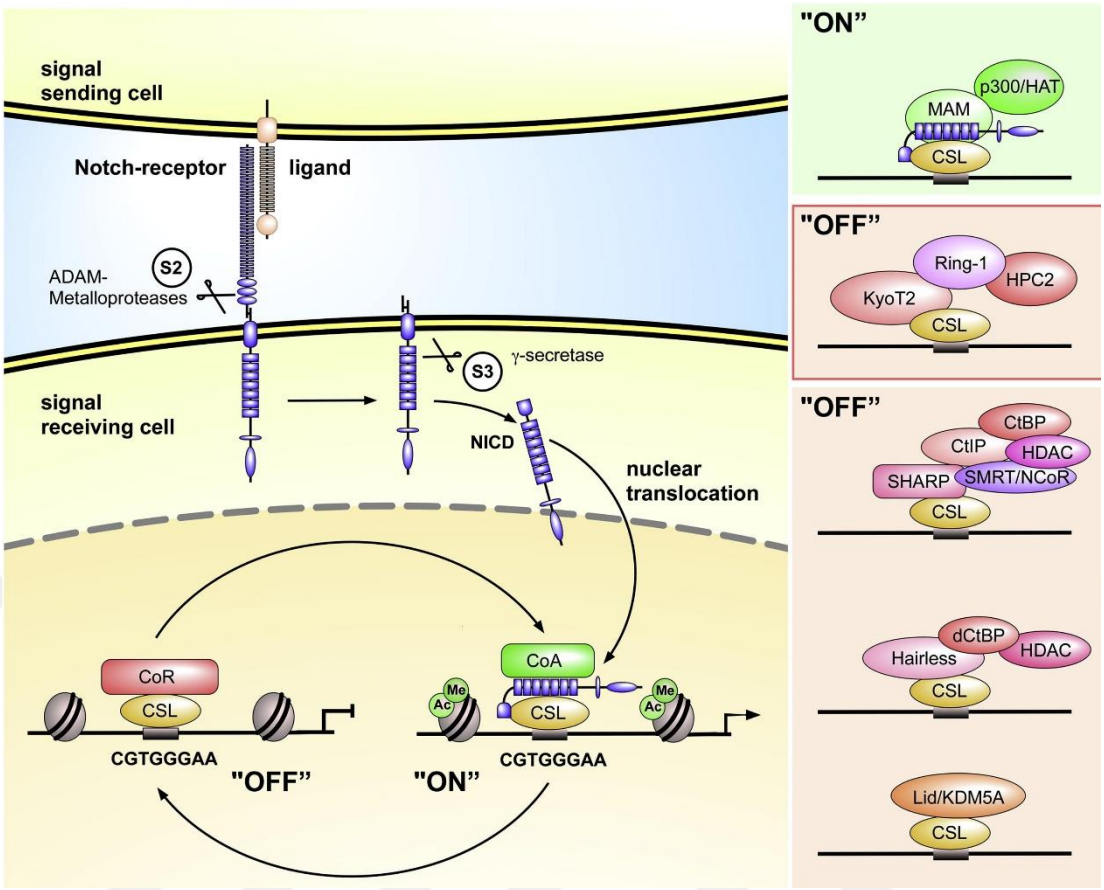


Figure 1.2: Molecular Steps Involved in Notch Signaling and Composition of the CSL/Notch Activator Complex and Different CSL-Repressor Complexes (Borggrefe & Oswald, 2014).

The moment NICD is released from the membrane via gamma-secretase, it relocates to the nucleus and interacts with CSL then builds up a trimeric complex with MAML-1 together with the additional co-activators. MAML-1 interacts with the ankyrin domain of NICD and stabilizes the interactions within the complex (L. Wu et al., 2000). This complex enables histone acetylation heading to the gene expression of the target genes. The Notch Transcription Complex is being regulated by MAML-1-p300 dimeric complex which acetylated the NICD itself and leads to the ubiquitin-dependent degradation of NICD (Borggrefe et al., 2016; Popko-Scibor, Lindberg, Hansson, Holmlund, & Wallberg, 2011).

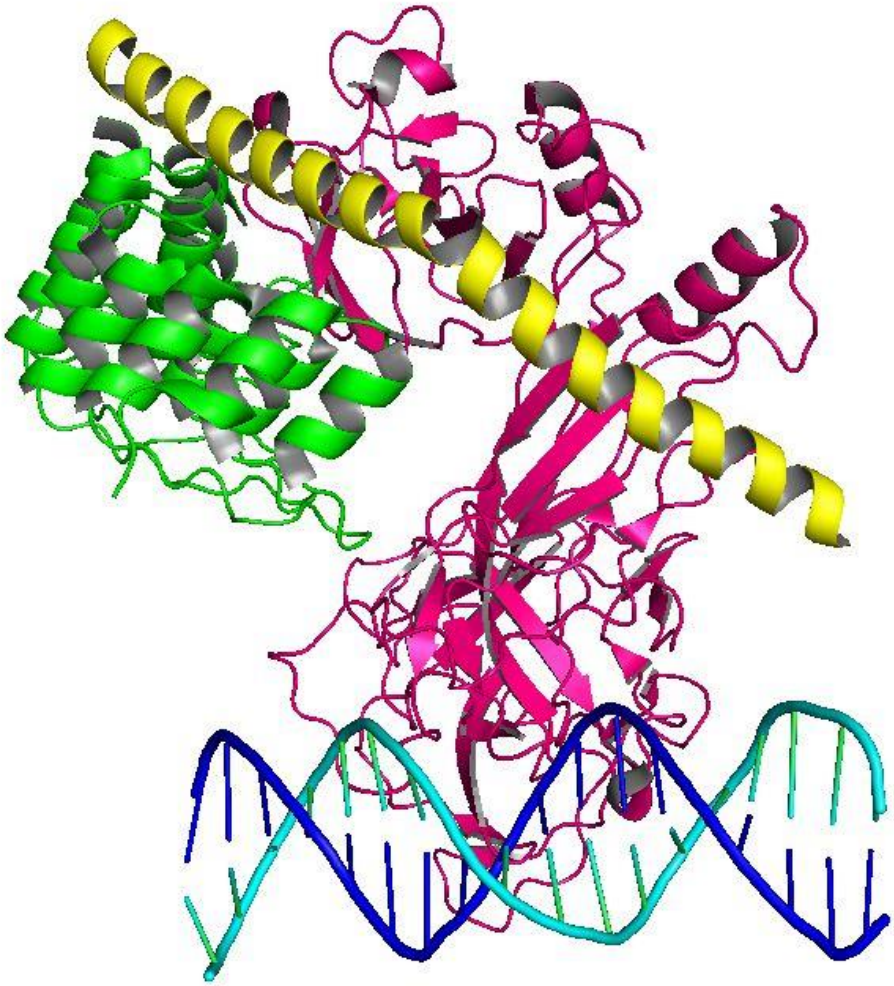


Figure 1.3: Interaction of NICD; CSL and MAML in Notch transcription complex on DNA (2f8x, PDB). The green chain represents Notch1-Ankyrin protein, yellow chain shows MAML-1 and pink chain is CSL protein.

Nam et al. reported the structure of the NTC, with one of the components of the complex being the ankyrin domain of human Notch1 (ANK). Because Notch signaling activates transcription, it regulates numerous cellular functions. However, the mechanism of selectivity for transcriptional activation of Notch1 is still not very clearly understood. Although the NTC can bind to DNA as a monomer, in some cases it is seen that it can also dimerize on accurately oriented and spaced DNA binding sites. The necessity of dimeric Notch transcription complexes was shown for T-cell maturation as well as leukemic conversion (H. Liu et al., 2010). It was discovered that some of the target genes such as c-Myc specifically require dimerized form while Hey1 or CD25 do not. This brings out the possibility of selectively influencing the expression of those genes by inhibiting the dimerized or monomeric version of Notch transcription activation complex

so that the outcomes resulted from those gene expressions such as Leukemia can be prevented (Abdel-Rahman, Martinez-Arias, & Blundell, 2011; Espinoza & Miele, 2013).

1.3.3. Role of Ankyrin Domain in Notch Transcription Complex

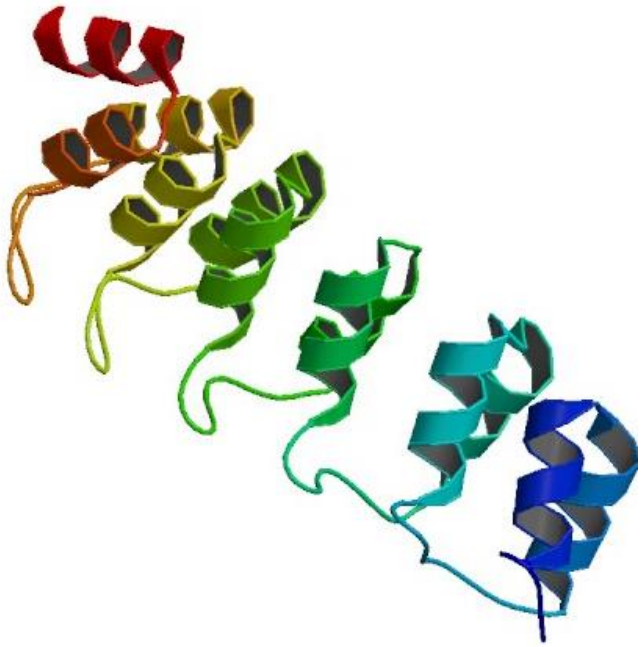


Figure 1.4: Crystal structure of Ankyrin protein in Notch intracellular domain(1yyh, PDB).

The intracellular notch domain contains seven ankyrin repeats.It is shown with site-directed mutagenesis studies that, ankyrin domain is influential in Notch signalling (Abdel-Rahman et al., 2011; Gordon et al., 2009) Gaining more data about the molecular dynamics of the each protein is very important since there is very few structural data that was not accompanied by a protein or DNA binding factor. It was shown that different protection patterns for the Ankyrin repeats where the repeats from four through six were the most protected ones (Kovall, 2012; Kovall & Blacklow, 2010). ANK domain is important for the assembly of the NTC in which the individual affinities of ANK and MAML-1 to CSL is very weak and ANK facilitates the formation of the complex (Kovall, 2012).

1.3.4. The Role of Notch1 protein in T-ALL

The Notch receptors take part in cell communication and regulation of cell growth and fate (Y. Wu et al., 2010). Notch also reacts to cell surface-bound ligands, hence it has a very important role during the differentiation process of the cells(Amsen, Helbig, & Backer, 2015). The abnormalities within the notch signaling pathways can be linked to various diseases, cancer being one of them (Palmer & Deng, 2015). It is suggested that Notch protein takes part at the differentiation of the mature T cells. T cells are required for protection against various microbial pathogens as well as the immune memory development (Amsen et al., 2015). Studies show that Notch regulates the th1 differentiation (Maekawa et al., 2003). Th1, known as T helper 1, is one of the CD4 T helper cells that produce specific cytokines to organize the immune system against different types of microbial organisms and viruses (Amsen et al., 2015; Basu, Hatton, & Weaver, 2013; Zhu, Yamane, & Paul, 2010).

T-cell acute lymphoblastic leukemia is an aggressive hematologic malignancy which is resulted from the oncogenic transformation of T cell progenitors, mostly seen in adults with the ratio of 25% and 15% of pediatric ALL studies (Stein et al., 2016; Van Vlierberghe & Ferrando, 2012). In about 50% of the T-ALL studies, activating mutations of Notch1 was observed which mostly seen in the heterodimerization domain(HD) and proline-glutamic acid-serine-threonine(PEST) domains (Stein et al., 2016; Weng et al., 2004). Given the high rate of mutation and the significant role of Notch signaling in T-ALL, Notch1 can be considered as a potential therapeutic target for the disease. In mouse model studies of T-ALL, it is also seen that inhibiting the Notch initiated significant anti-leukemic effects in vivo(Stein et al., 2016; Tatarek et al., 2011).

1.3.5. Therapeutic studies against Notch Transcription Complex

Notch receptors are transmembrane proteins that take part at transmitting juxtacrine signals which are initiated by the ligands called Delta, Lag-2 or Serrate family. Ligand binding activates ADAM and gamma-secretase proteolytic activity resulting in the relieve of Notch intracellular domain which also carries ankyrin repeats on it (De Strooper et al., 1999; Moellering et al., 2009; Struhl & Greenwald, 1999). Notch intracellular domain then relocates to the nucleus and binds to CSL, this interaction with CSL creates a long,

shallow groove along the interface of the two proteins which then acts as a binding surface for MAML-1(L. Wu et al., 2000).

There have been a couple of studies which aimed to disrupt the interaction of the Notch transcription complex proteins. Moellering et al. decided to target Notch Transcription complex via hydrocarbon-stapled alpha-helical peptides against the dominant negative fragment of MAML-1(dnMAML1) and called them SAHMs(stapled alpha-helical peptides derived from MAML-1). It was observed that SAHM1 inhibited the binding of MAML-1 to CSL-ANK complex competitively. Also, after being treated with SAHMs, T-ALL cells showed a decrease in the expression of HES1, MYC and DTX1 which are among the Notch1 target genes(Moellering et al., 2009).

In another study, in order to disrupt or stop the release of intracellular notch1, small inhibitors against gamma-secretase complex was used which resulted with not just disrupting the Notch pathway, it also affected the other downstream pathways which were interacting with the gamma-secretase complex(Dovey et al., 2001; Lleo, 2008). Selectivity within drugs is very important for an effective disease treatment. Selectivity is also a very challenging trait to achieve with the proteins like Notch, which is very broad so that it is hard to figure out the structure for designing small molecules which will specifically bind to the Notch then prevent the Notch Transcription complex assembly. There are many studies still going on in industry and academia to figure out this problem and find a target that will specifically bind to Notch Transcriptional Complex and prevent it from transcription or disturb the complex.

1.3.6. X-Ray Crystallography

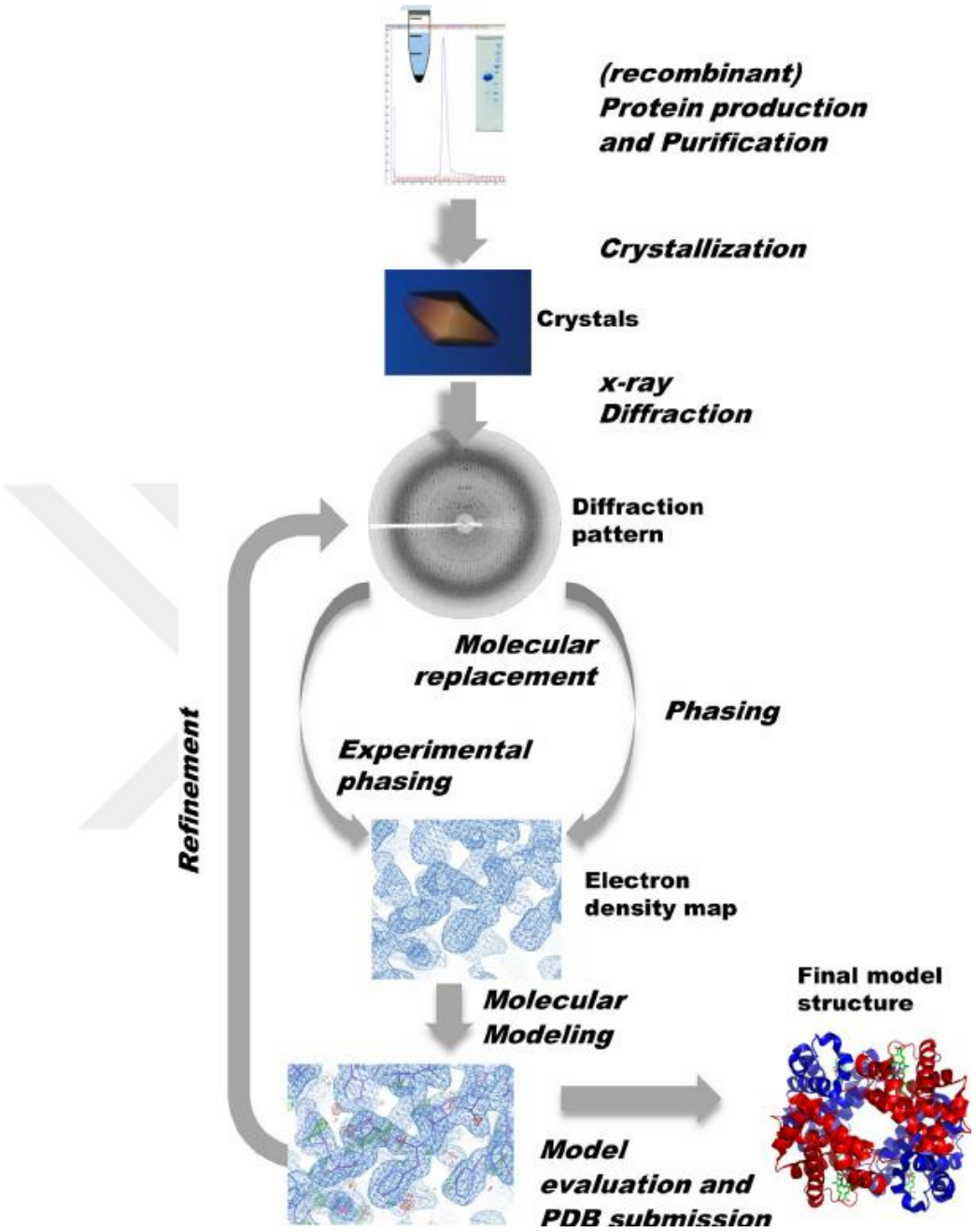


Figure 1.5: Workflow of X-ray crystallography. Workflow for macromolecular structure determination by X-ray crystallography (Molecular Biology of the Cell, 2002).

Three-dimensional structures of biologic macromolecules are used for figuring their function so that it is a very important field of natural sciences and medicine. The findings of the atomic resolution structures of a molecule provide scientists with a deep understanding regarding the function of the protein while helping them to solve its role

in the living cell. About 86% of the Protein Data Bank(rsb-PDB) entries are macromolecule structures which were determined via X-Ray crystallography. To be able to get crystals that can be used for crystallographic studies, the macromolecule must be purified to homogeneity or be close to it, the homogeneity is one of the key factors for obtaining crystals which diffract well and give high-resolution data (McPherson, 2004).

Crystallization requires bringing the macromolecule to supersaturation. The sample should be concentrated to the highest possible concentration without causing aggregation or precipitation of the macromolecule. Introducing the sample to a precipitating agent can promote the nucleation of protein crystals in the solution, which can result in large three-dimensional crystals growing from the solution.

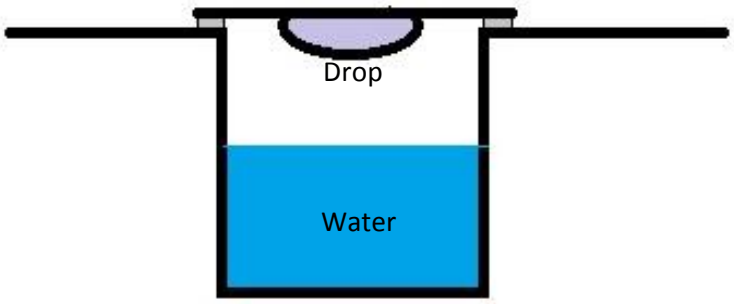


Figure 1.6: Hanging drop vapor diffusion

The most common method used for protein crystallization is called *hanging drop vapor diffusion.* In this method, a purified and concentrated protein solution is mixed with a precipitant and left to equilibrate via evaporation. Under optimal conditions and right precipitant, a protein crystal formation will happen. In this method, a small volume of protein and precipitant are combined on a glass coverslip and sealed over a well which contains reservoir solution. Since the precipitants concentration in the drop will be lower than in the good solution, water evaporates from the drop and increases the concentration of both protein and precipitant, causing equilibrium with the good solution which this condition then favor crystal formation instead of precipitation.

High-throughput screening of fragments using crystallography requires fast and efficient X-ray data collection, either in-house or at a synchrotron radiation source. Recent developments in hardware caused the need to reorganize and advance data collection at

synchrotron beamlines where new third generation sources, producing brighter and better-collimated X-ray beams, allow higher quality data to be composed rapidly (Blakeley, Cianci, Helliwell, & Rizkallah, 2004; Davies & Tickle, 2012).

1.3.7. Fragment-Based Drug discovery

Fragment-Based Drug Discovery(FBDD) is based on the idea of a small fragment which can be identified and then either grown, merged, or linked with another fragment to improve efficiency. Hence, the fragment needs to be small enough to be able to prevent molecule creation which is too large to be useful as drugs (Davies & Tickle, 2012; Erlanson, 2012). Fragment-based drug discovery can be described as the finding of drugs via using fragments or data resulting from the fragments which were not discovered by traditional methods (Erlanson, 2012). Crystallography and protein detected NMR are techniques that are used in providing detailed actual information on how ligands bind to proteins (Erlanson, 2012).

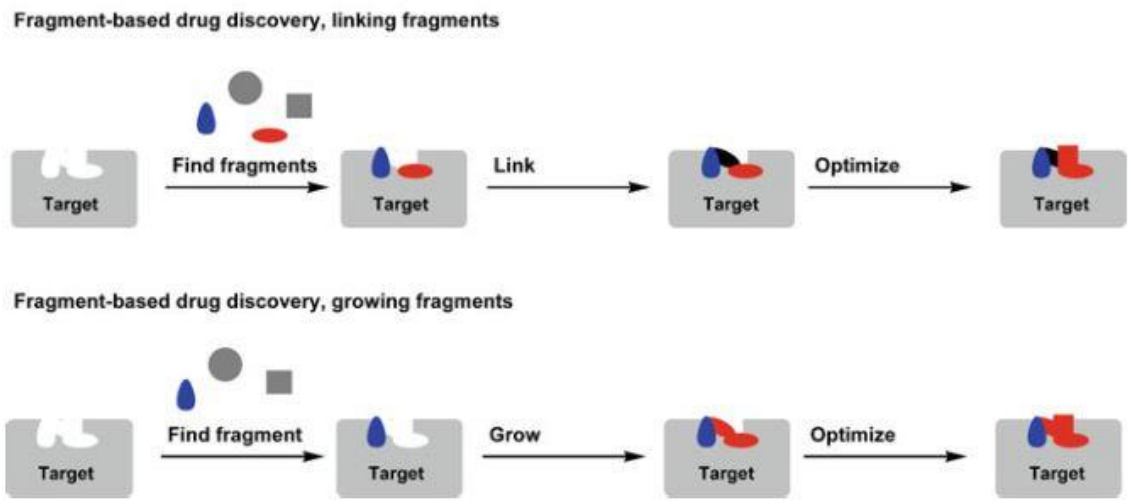


Figure 1.7: Representation of fragment linking and fragment growing(Erlanson, 2012)

Fragment-Based Drug Discovery showed a rapid increase over the 15 years correlated with throughput crystallography. There are companies that use FBDD with crystallography or some of them only work with the fragments that can be characterized via crystallography (Erlanson, 2012).

Soaking the ligand of interest into apoprotein crystals is the most effective method for obtaining a protein-ligand complex via crystallography which usually achieved by

transferring a single crystal into a high concentration of ligand for a time being by allowing the drug diffuse into the channels in the crystal then bind at energetically favorable places (Davies & Tickle, 2012).

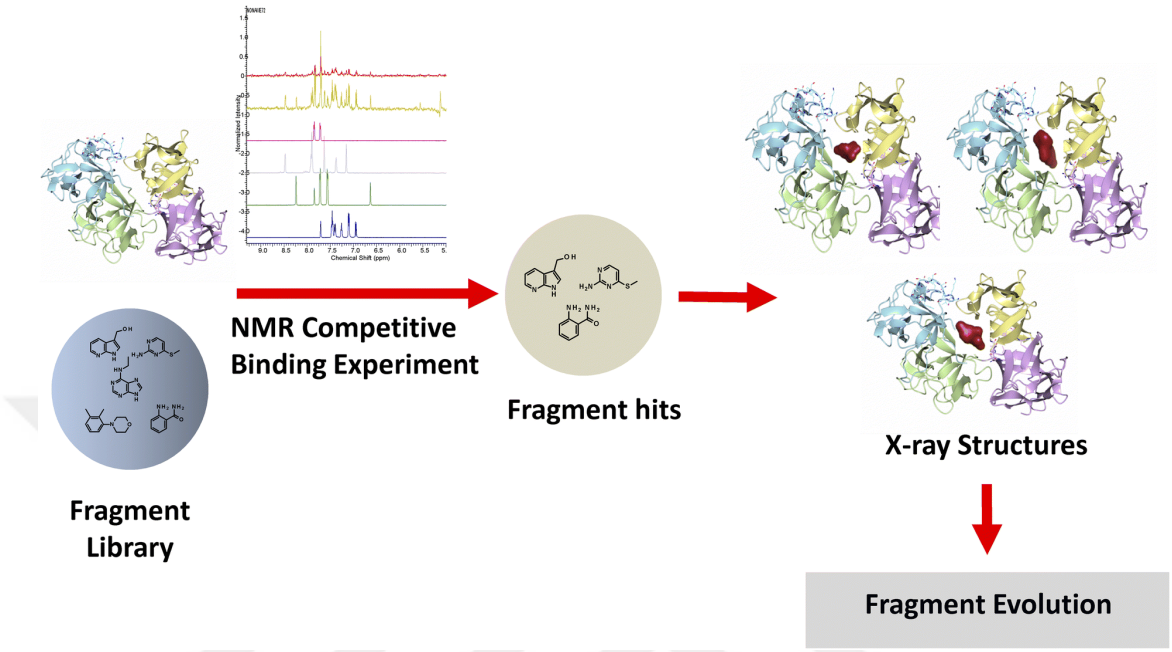


Figure 1.8: Fragment-Based Drug discovery (Drysdale, 2015).

Another procedure for obtaining protein-ligand complexes called co-crystallization. In this procedure, Protein-ligand complex is prepared in a liquid phase and so proteins are crystallized together with the ligand. This procedure is not always favored since the addition of ligand can change the crystallization conditions which then can cause a set of experiments to figure out a new condition for crystal formation. Furthermore, co-crystallization is not optimal for detecting weakly binding fragments because the very high concentration of ligand is needed to fully occupy the binding site can also interfere with the crystallization process itself (Davies & Tickle, 2012). In addition, co-crystallization can be used in cases where fragment soaking causes crystals to crack, probably causing conformational changes or binding at crystal contacts.

2. MATERIALS AND METHODS

In this study, we aimed to gain more information regarding the 3D structure of Ankyrin protein which resides among Notch1 human protein domains while taking part in Notch Transcription Complex assembly in cells. We designed two ANK protein fragments which were shortened from their C- and N-terminus expressed them via recombinant protein expression technology, purification was employed by using Affinity Chromatography and Size Exclusion Chromatography. The purity of proteins was checked via SDS-Page Electrophoresis. Purified proteins were used for initial screening in order to decide conditions for crystal formation and growth. Protein crystals were produced under optimized conditions and their structures were determined by using X-ray diffraction in-house and at SLS. In order to check the interaction of chemical compounds/fragments with ANK domain, we used fragment-based drug discovery. We prepared several conditions to check Protein-fragment interaction and tried to produce protein crystals with proposed chemical fragments for X-ray diffraction to observe the absence of an abundance of the interaction.

2.1. RECOMBINANT EXPRESSION AND PURIFICATION OF ANKYRIN PROTEIN FRAGMENT

2.1.1. Chemical Transformation of Vector into *E.Coli*

Chemically competent *E.Coli* BL-21 (DE3) from -80 C were used as a host for transformation. GST-Tagged Notch1 protein sequence containing ampicillin resistant vectors pGEX6P-2_GST_NOTCH1_ANK_T1927-G2126_TEVsite (5605bp) and pGEX6P-2_GST_NOTCH1_ANK_E1929-G2126_TEVsite (5599bp) were transferred to the *E. Coli* BL-21 (DE3) cells for protein expression and cells were plated in LB-Amp plates for overnight at 37°C for growth (Vector data in Appendice). For transformation, we first thawed *E. Coli* BL-21 cells which were taken from -80°C and transferred 10µl 0of cells into the new Eppendorf tubes. Following the addition of 1 µl plasmid, the tubes were kept on ice for 30 minutes. Then incubated on the heater at 42°C for 45 seconds and were kept on ice for 2 minutes. After that, pre-heated (at 37°C) SOC medium was added to the cells and kept on thermomixer (Eppendorf Thermomixer comfort), at 37°C for 1

hour, shaking. We then spun down the cells at 5.000 rpm at Eppendorf Centrifuge 5417R for 2 minutes. The supernatant was discarded, then the pellet was resuspended and inoculated on Ampicillin containing LB plates, then incubated at 37°C, overnight, for growth.

Table 2.1: The list of the mediums used for transformation, bacterial cell culture, and protein expression.

Name	Compounds
SOC Medium	5g Yeast-Extract, 20g Tryptone, 0.58g NaCl, 0.19KCl, 2.03g $MgCl_2$ x 6 H_2O, 2.46g $MgSO_4$ x 7 H_2O, Add 960ml H_2O; Autoclave all, then add 50% sterile Glucose.
LB Medium	5g Yeast-Extract, 10g Tryptone, 10g NaCI, Add 1 L H2O(pH:7,4).
TB Medium	Solution 1: 24g Yeast-Extract, 12g Tryptone, 4ml Glycerol, add 900ml H_2O and Autoclave. Solution 2: 0.17*M* KH_2PO_4, 0.27*M* K_2HPO_4, add 90ml H_2O. Then mix Solution1 and Solution 2 together.

2.1.2. Recombinant Expression of Ankyrin Proteins

Pre-culture was prepared by using Ampicillin containing TB liquid Media. 200µl Ampicillin was added to the 200ml of TB media. A vector containing cells were transferred to the pre-culture media by using the inoculation loop. Cells were kept overnight at 37°C. The next day, total 5 liters of TB/Amp+ media was prepared. Cells were transferred from the pre-culture into 2,5 liters of TB/Amp+ media containing flasks and left to grow at 37°C, shaking at 140 rpm. After the cell culture reached to an OD around 0.6 which measured via Nanodrop 2000C Spectrophotometer, cells were inoculated by adding 0.5*M*, 500µl IPTG with the final concentration of 0.1*mM*. Then proteins were expressed at 25°C, shaking at 170 rpm, overnight. Cells were spun down via Backman JA 25.50 centrifuge at 4000 rcf for 45 minutes. Pellets were kept and resuspended in TBS buffer up to final volume of 200ml. PMSF was added to the cells, l µl per ml. Then followed by the addition of DnaseI(Bioline) and Lysozyme(Sigma-Aldrich). All mixed by magnetic stirrer and cells were further lysed via microfluidizer (Microfluidics Corporation) by using wash buffer (50m*M* Tris, 150m*M* NaCI, 1m*M*

EDTA, 2m*M* DDT), then kept at -20°C, later on, to be used for affinity purification and TEV digestion.

2.1.3. Affinity Chromatography

Affinity chromatography is a method that is used for separation of molecules by using their specific interactions. It is based on the use of an affinity ligand coupled to a matrix which lets specific capture of the product from a mix. By using this method, a pure product can be obtained (Uhlen, 2008)

In this study, GSH column was used which shows high binding affinity against GST protein that is being used as Tag for Ank for the study. The GST-Tagged protein containing cell lysates were previously separated from cell lysate with affinity chromatography and protein mixture was shock-frozen via liquid nitrogen. For separation of GST tag from Ankyrin protein fragments, affinity chromatography was employed. First, The proteins were thawed and protein digestion was performed by using TEV protease (DPF-Max Planck Institute, Dortmund) which had activity under the conditions of 0.05mg TEV protease per 1mg of protein. 514.3µl of TEV-Protease was mixed with 144mg of GST-Tagged Ankyrin proteins. The protein mix kept at 4°C for overnight for digestion.

In the meantime, 10µl Protein samples were taken before and after cleaving process as well as at each step during chromatography for SDS-PAGE gel running. One liter of wash buffer and 250ml of Elution buffer were prepared and kept at 4°C. Wash buffer contained 50m*M* Tris, 150m*M* NaCI, 1m*M* EDTA, 2m*M* DDT and elution buffer contains 50m*M* Tris, 150m*M* NaCI, 1m*M* EDTA, 2m*M* DDT, 20m*M* GSH, both buffers adjusted to final pH:8.0. The column was run with water then equilibrated with wash buffer equal to one column volume (which also depends on the size of the column) with 1.5 ml per min flow rate, by using a peristaltic pump (Ismatec MCP process IP65). Digested proteins were loaded onto the column, cleaved proteins were washed from the column after loading two column volume of wash buffer, Remaining GST-Tags from the column were removed from the column using elution buffer. The proteins were concentrated via Amicon ultra-15 Centrifugal filter devices depending on protein size and volume, 30K or 10K and in 50ml or 500µl of volumes, respectively

2.1.4. Size Exclusion Chromatography

In general, separation of molecules such as proteins is decided via the size of the molecules in the solution. This size-dependent separation allows a calibration curve in principle, which results from the known molecules in the solution and it can also be used for the estimation of the molecular weight of the unknown molecule in the solution (Hong, Koza, & Bouvier, 2012).

Following affinity column purification, size exclusion chromatography was performed for each protein fragments by using HPLC or FPLC(GE Akta Explorer, GE Akta FPLC, respectively). Gel filtration is a method where proteins are being separated via their molecular weight and samples in the column are being separated into fractions.Before performing gel filtration, first, 10X concentrated buffer was prepared. Then diluted to 1X and DDT was added every time before running the column. 10X buffer contained 0.5*M* Tris, 1.5*M* NaCI, final volume 2.25L with a pH:8.0 then stored at 4°C. 2m*M* DDT was added to the 1X buffer then degassed every time before use. Approximately 20-25mg of proteins were loaded onto the column. In the end, proteins were separated into fractions which then kept at 4°C for SDS-PAGE analysis.

2.1.5. SDS-PAGE Electrophoresis

Protein expression, digestion and column purification results were checked via SDS-PAGE gel electrophoresis. SDS-PAGE gels were prepared with the concentration of 12% by using Bio-rad SDS gel kit(Bio-Rad Mini-PROTEAN Tetra Cell) (Appendices). 10µl of proteins from each step before, during and after purifications and digestion were taken and mixed with 20µl of 5X SDS Loading buffer and kept at 90°C for 3-5 minutes for boiling, then 5µl of protein from each sample loaded to the gel respectively and SDS-PAGE gel was run at 90V for 1h by using. Gels were stained by using coomassie blue staining (40% Ethanol, 0.3% Coomassie Blue R250, 0.3%Comassie Blue G250), then microwaved about 1 min. and kept in staining solution for 30 minutes, shaking. After then, the gel was washed with destaining buffer for 1h., the buffer exchanged with water and gels were kept in water overnight, shaking, for further destaining.

2.2. X-RAY CRYSTALLOGRAPHY

2.2.1. Initial Screenings

Purified 5mg/ml, 8mg/ml and 10mg/ml of ANK E1929-G2126 and ANK T1927-G2126 protein fragments were used for initial screenings settings. For initial screening set up, JCSG Core Suites(I-IV) were used which split to 4 different 96 unique conditions. Those 398 screens provide most hits among the other crystallization conditions. For Initial Screening 96-weel plates were used(96-Well Sitting-Drop IQ Plates) from TTP lab tech and crystal drops were set by using TTPLabtech Mosquito LCP with the ratio of 1:1. Prepared initial screenings were put into Formulatrix Rock Imager 1000 for incubation and crystal formation.The formulatrix was set for room temperature and 4°C and took images of the drops in initial screening plates on certain days that was set up via formulatrix software.

2.2.2. Optimization of Crystal Growth Conditions

Depending on the protein crystal formations on initial screening results, refinement conditions were decided and buffers were prepared. 24 well plates were used for crystal setups called Crystalgen 24 Well SuperClear^{TM} Plates (Jena Bioscience). Hanging drop technique was used during the protein crystallization setups. The proteins were mixed with the reservoir solution ratios at 1:1, 1:2 and 1:3 respectively. The ratio between of protein and reservoir solution mix varied from 0.5µl to 3µl as well as reservoir amount which also varied from 500µl, 700µl, and 900µl respectively.

2.2.3. Protein Crystal Refinements

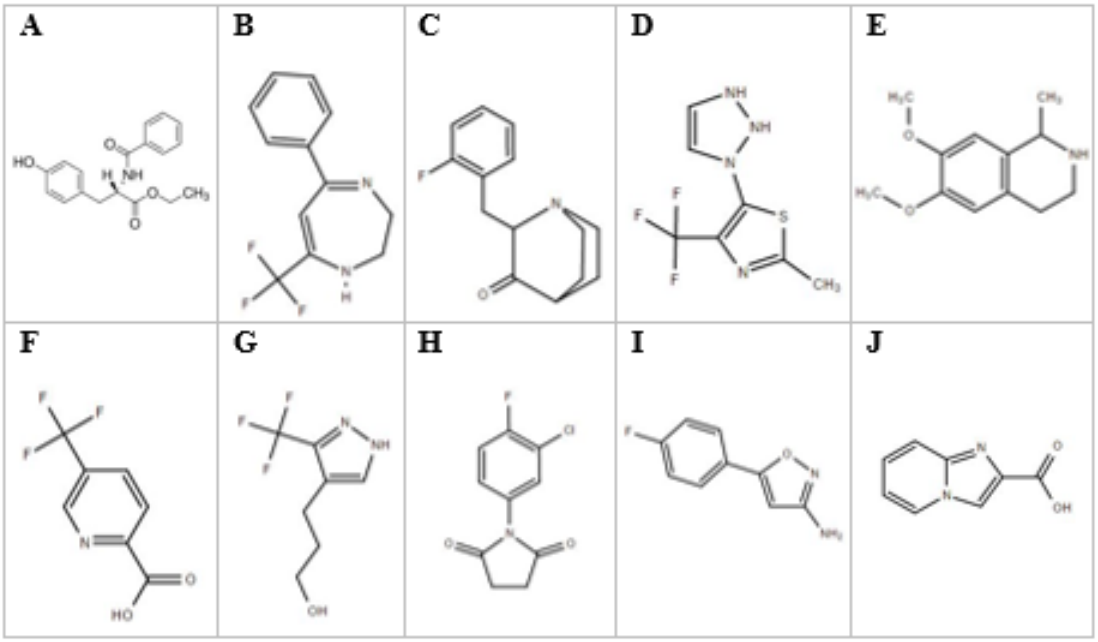
Protein-reservoir mixes were set up as hanging drops in 24 well plates and kept at 4°C to get bigger, fishable and more stable crystals which in the end provided diamond shaped crystals size between 50-100micron. Protein drops were checked under the microscope (Leica M125, Olympus CX41) for any protein crystal development or the growth of the already formed protein crystals in the drops.

2.2.4. Fragment-Protein Interaction

Ten chemical fragments(compounds) which were detected via NMR were used for protein-ligand interaction detection (Table 2.2). ANK E1929-G2126 was mixed with the saturated fragments solutions which were prepared in 1% DMSO as well as 1% DMSO

and reservoir solution of the designed condition, with the ratio of 1:1,1:2,1:3 during initial screenings. Selected conditions were used for further protein-ligand crystal growth in 24-well plates with same conditions that applied to the apoprotein fragments in the beginning.

Table 2.2: Chemical compounds used for fragment-based drug discovery assay. **A:** N-Alpha-Benzoyl-L-Arginine Ethyl Ester·HCl. **B:** 5-Phenyl-7-(Trifluoromethyl)-2,3-dihydro-1H-1,4-Diazepine. **C:** 2-(2-Fluorobenzyl)-3-Quinuclidinone. **D:** 3-[2-Methyl-4-(trifluoromethyl)-1,3-Thiazol-5-yl]-1H-1,2,3-Triazole. **E:** 6,7-dimethoxy-1-methyl-1,2,3,4-tetrahydroisoquinoline hydrochloride. **F:** 5-(Trifluoromethyl)Pyridine-2-Carboxylic Acid. **G:** 3-[3-(Trifluoromethyl)-1H-Pyrazole-4-yl]-1-Propanol. **H:** 1-(3-Chloro-4-Fluorophenyl)dihydro-1H-Pyrrole-2,5-Dione. **I:** 3-Amino-5-(4-Fluorophenyl) Isoxazole. **J:** Imidazo[1,2-a]Pyridine-2-Carboxylic Acid Monohydrate.



2.2.5. X-Ray Diffraction and Structure Analysis

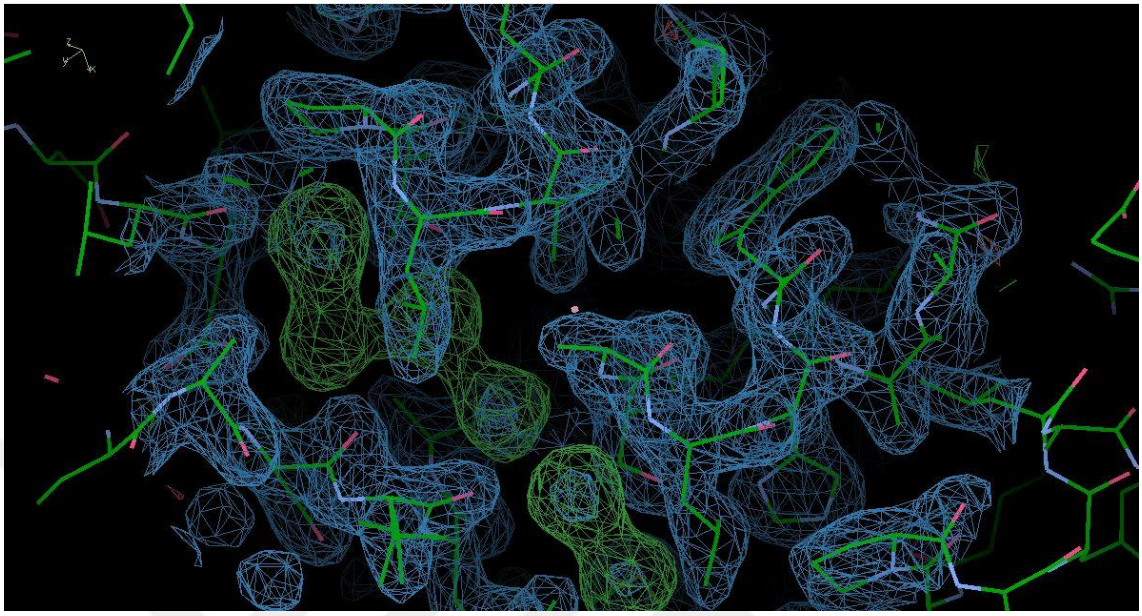


Figure 2.1: Electro density map by using the input from X-ray diffraction data (WinCoot, ver. 0.8.4).

Protein crystals were fished with using cryo loops. As for cryoprotectant solution, 30% glycerol was used for apoprotein crystals though for ligand-protein co-crystals which was prepared by using gel filtration buffer ending up with 33% of glycerol that was prepared with saturated fragment solution with 1:2 ratio (2µl glycerol: 4µl saturated fragment buffer). Several Protein crystals were fished via using crystal loops and stored in liquid nitrogen for X-Ray diffraction. Diffraction data of the protein crystals was solved via CCP4i (*ver.* 7.0.031), proteins were built via WinCoot (*ver.* 0.8.4) and Pymol (*ver.* 4.20) software.

3. RESULTS

Two protein fragments, ANK T1927-G2126 and ANK E1929-G2126 were expressed in *E.Coli,* purified and characterized via Column and Size Exclusion Chromatography, SDS-Gel analysis. Cloning of the both vectors was done by Sebastian Kiehstaller(Appendices). Then these two purified protein fragments were used for optimizing the conditions for crystal growth then checking the diffraction of the protein crystals via X-ray crystallography and employed for the fragment-based drug discovery

3.1. EXPRESSION, PURIFICATION, AND CHARACTERISATION OF ANKYRIN PROTEIN FRAGMENTS

3.1.1. Expression, Purification, and Characterisation of ANK T1927-G2126

NICD-ANK T1927-G2126 was expressed by using *E. Coli* as a host organism. Expressed protein mix was run at GSH Column to get the GST-ANK T1927-G2126 protein then digested by TEV Protease overnight and Purified by using GSH Column again which separated GST and ANK T1927-G2126 (Figure 3.1). In order to get the much pure protein, size exclusion chromatography was employed. Proteins were separated by their size to the fractions. In the end, we collected the fractions with the ANK T1927-G2126 (Figure 3.2) and used them for initial screenings.

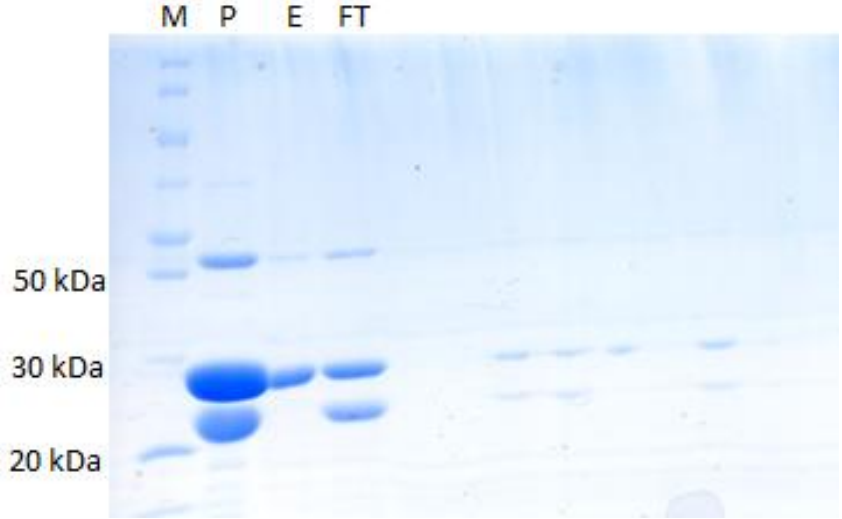


Figure 3.1: SDS-Gel analysis of the NICD ANK T1927-G2126 Protein fragments. TEV-digested and GSH Column purified protein that runs on 12% SDS Gel. M; marker, P; Digested protein (GST, ANK), E; elution of the column, FT; Protein fragments purified via column.

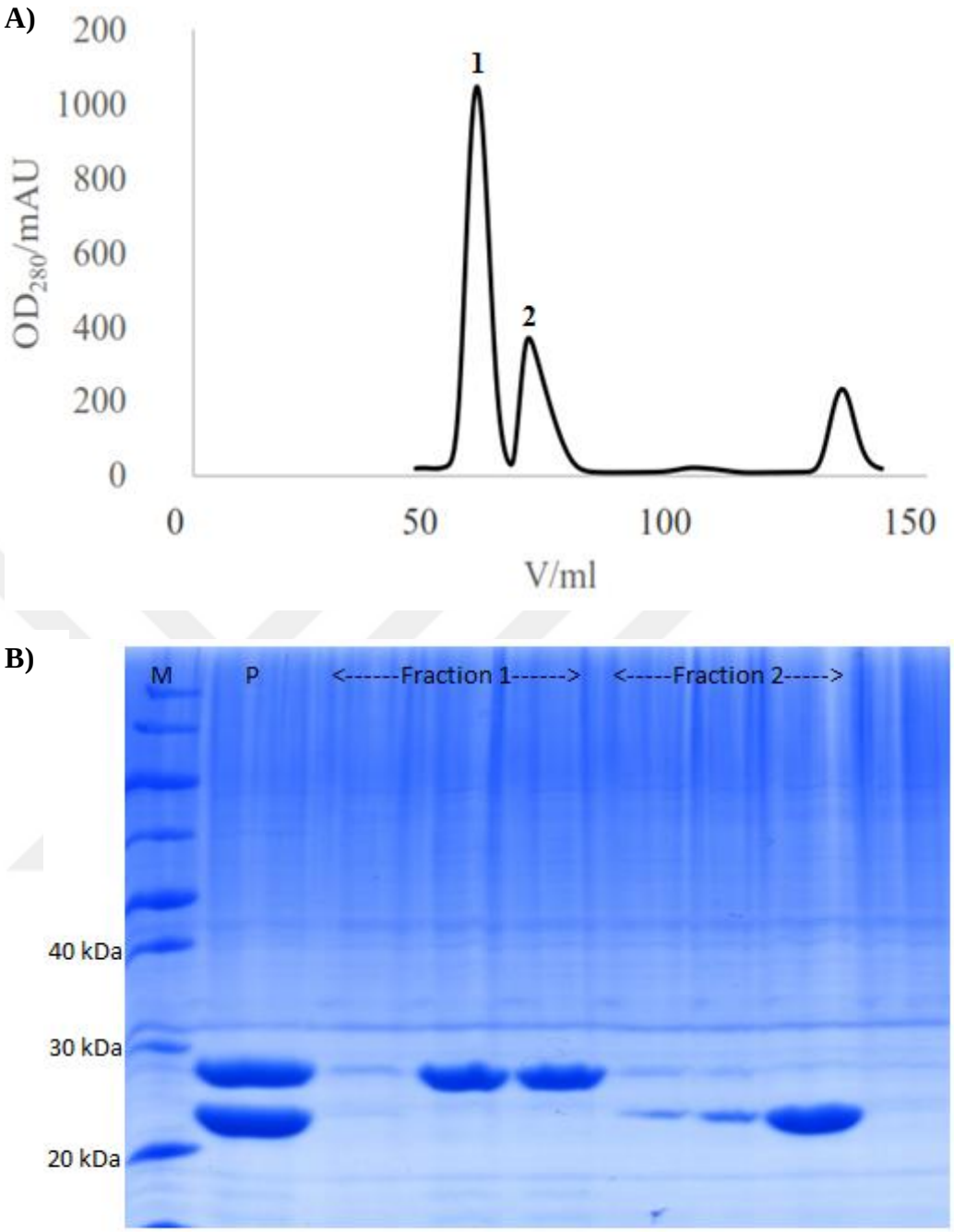


Figure 3.2: Size exclusion chromatography results of previously GSH column purified proteins. A: Size exclusion purification, protein fraction data, first peak shows GST-Tag protein, the second peak shows ANK T1927-G2126 protein fragment. B: SDS-Gel characterization of the protein fragments following size exclusion chromatography. Protein samples loaded and run at the SDS-Gel (12%). M: Marker, P: GSH Column purified, TEV digested GST-TEV and ANK T1927-G2126 protein fragments. Fraction 1 is GST Tag protein and Fraction 2 is ANK T1927-G2126.

3.1.2. Expression, Purification, and Characterisation of ANK E1929-G2126

NICD-ANK E1929-G2126 was Expressed in *E. Coli* (DE3) and collected cells were lysed before purification process. Lysed protein mix was then run at GSH Column to get the GST-TEV-ANK E1929-G2126 protein then digested by TEV Protease overnight and column purified by using GSH Column which separated GST and ANK E1929-G2126. In order to get the protein pure enough for crystallization, size exclusion chromatography was employed. Proteins were separated by their size into the fractions. In the end, we collected the fractions with the ANK E1929-G2126 and run them on 12% SDS-Gel for further characterization(Figure 3.3 and 3.4). Then purified proteins were used for initial screening set-ups, refinements, and co-crystallization experiments.

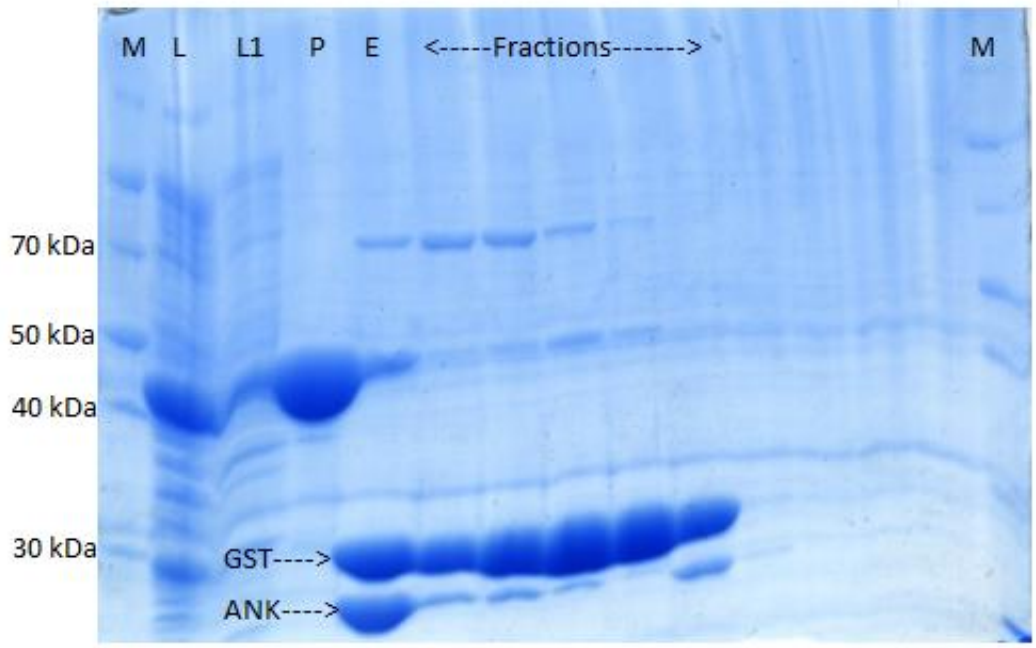


Figure 3.3: SDS-Gel analysis of the NICD ANK E1929-G2126 Protein fragments. Undigested, TEV-digested and GSH Column purified protein that run on 12% SDS Gel. M; marker, L: Cell lysate, L1: protein in supernatant before column purification, P; Undigested protein, E; elution of the digested protein from the column, Fractions; Proteins purified via gel filtration.

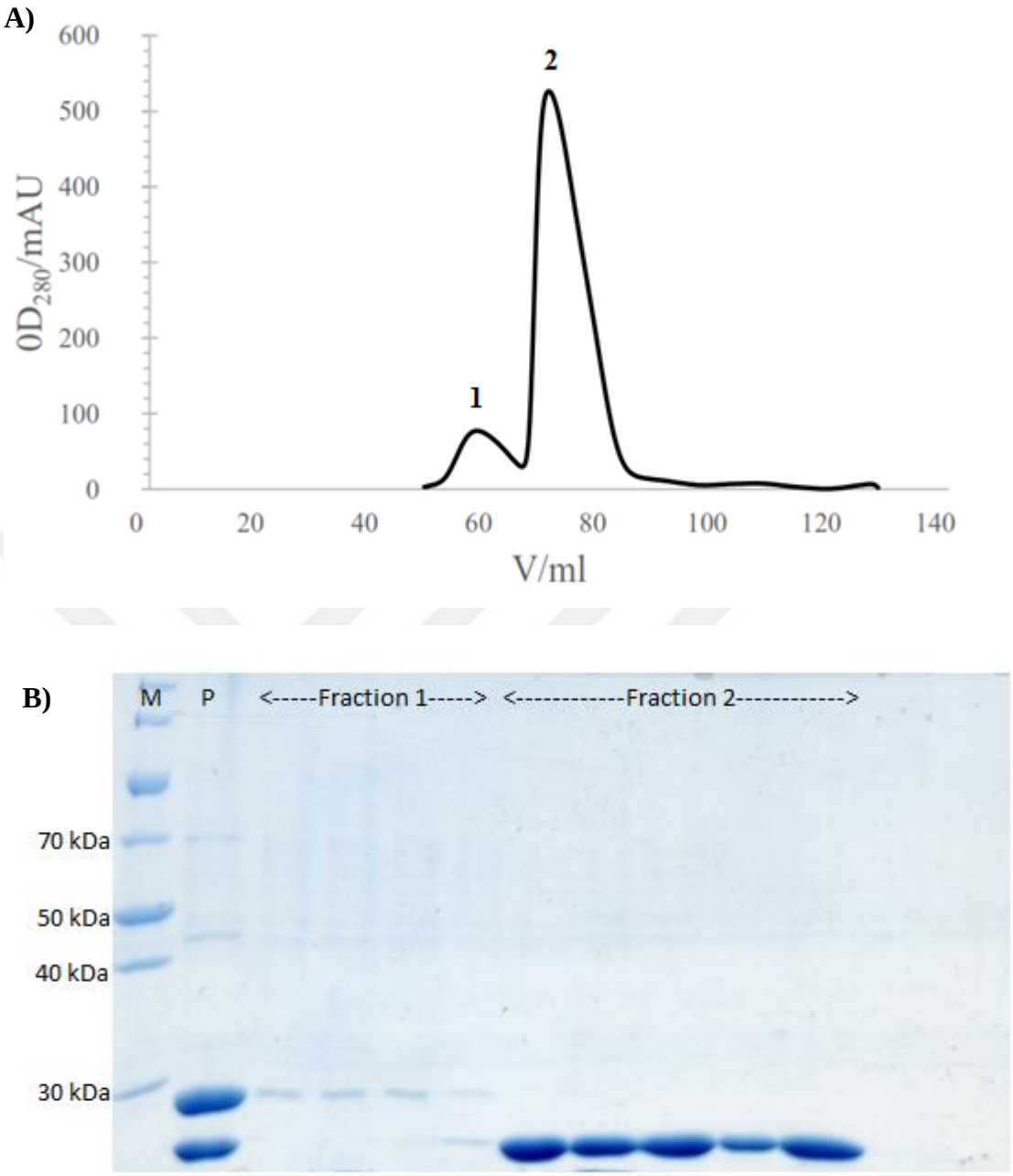


Figure 3.4: Size exclusion chromatography results of GSH column purified ANK E1929-G2126. A: Size exclusion purification, protein fraction data, first peak shows GST-Tag protein, the second peak shows ANK E1927-G2126 protein fragment. B: SDS-Gel characterization of the protein fragments following size exclusion chromatography. Protein samples loaded and run at the SDS-Gel (12%). M; Marker, P: TEV digested GST and ANK E1929-G2126 protein fragments. The first fraction is GST Tag protein and the second fraction is ANK E1929-G2126.

3.2. X-RAY CRYSTALLOGRAPHY

3.2.1. Initial Screenings of ANK T1927-G2126 and ANK E1929-G2126

Initial screenings were set up and put into Formulatrix Rock Imager 1000 for incubation at certain temperatures as well as following any crystal formation or growth in the drops. There were several conditions set up in the process. Firstly, initial screening of ANK T1927-G2126 and ANK E1929-2126 apoproteins were set up, respectively, via 96 well plates in 4C degree and room temperature. Then images were taken by the rock imager on set days.

Most of the crystals were formed at 4C degree where there were only a couple possible crystallizing conditions in RT which did not give any crystals. In Figure 3.1, ANK T1927-G2126 apo-protein crystals were formed. Both conditions were refined and proteins formed smaller crystal pieces (crystal seeds even) which were not fishable for further investigation (Figure 3.9).

The second initial screening was prepared using ANK E1929-G2126 apo-protein. In comparison to ANK T1927-G2126, ANK E1929-G2126 protein formed more defined, stable and fishable crystals. It formed several crystals under different buffer conditions in both initial screening and refinement settings (Figure 3.6).

The ten fragments (Table2.2, which was selected by NMR and had potential to interact with ANK Domain, was dissolved in 1%DMSO. By using saturated fragment solutions, another refinement was set up. Since ANK E1929-G2126 more crystals with different buffers at 4C degree, we set up new screening to find the conditions that gave crystals with protein and fragment combined. Then we got crystals with compound I (Figure 3.7).

A)

B)

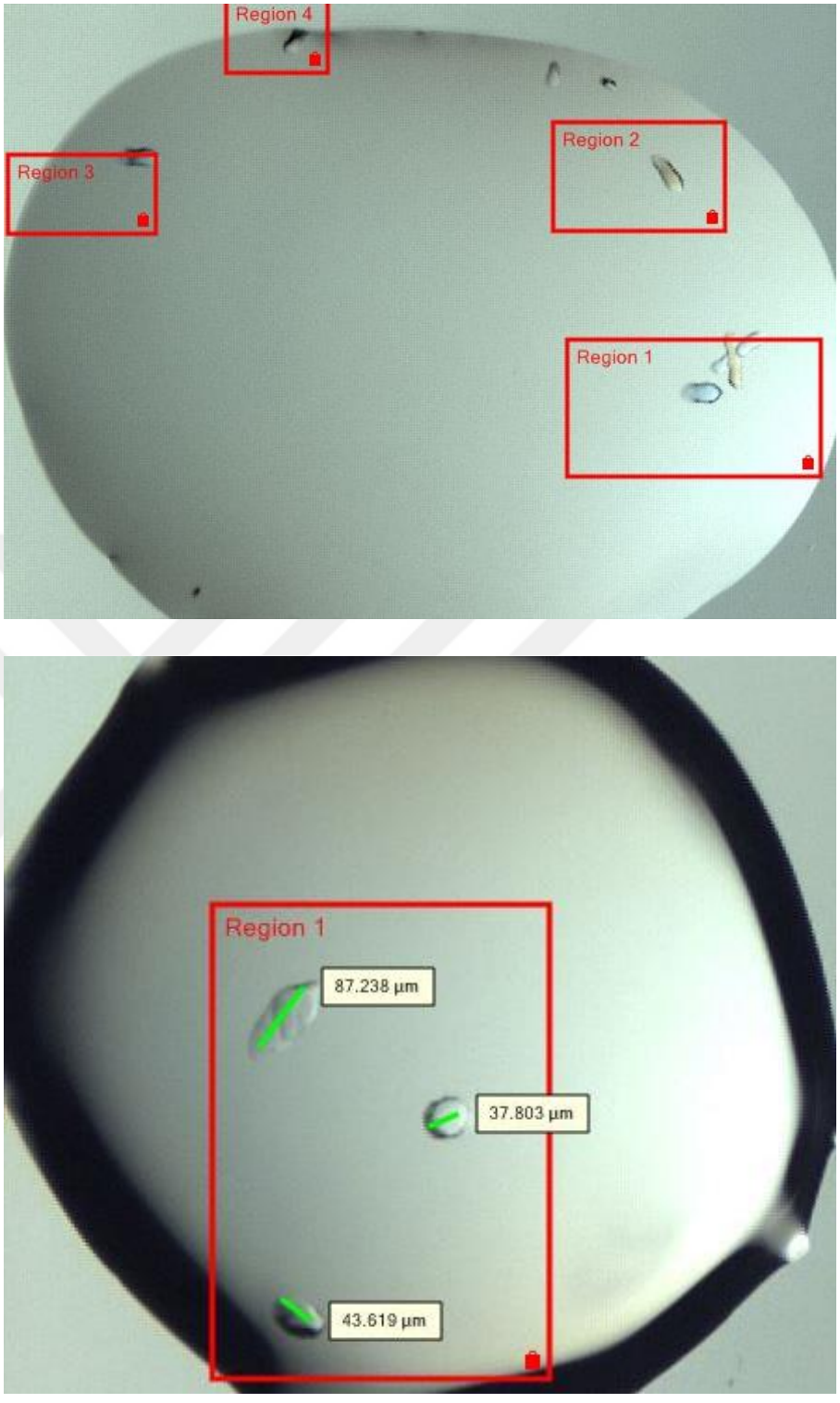
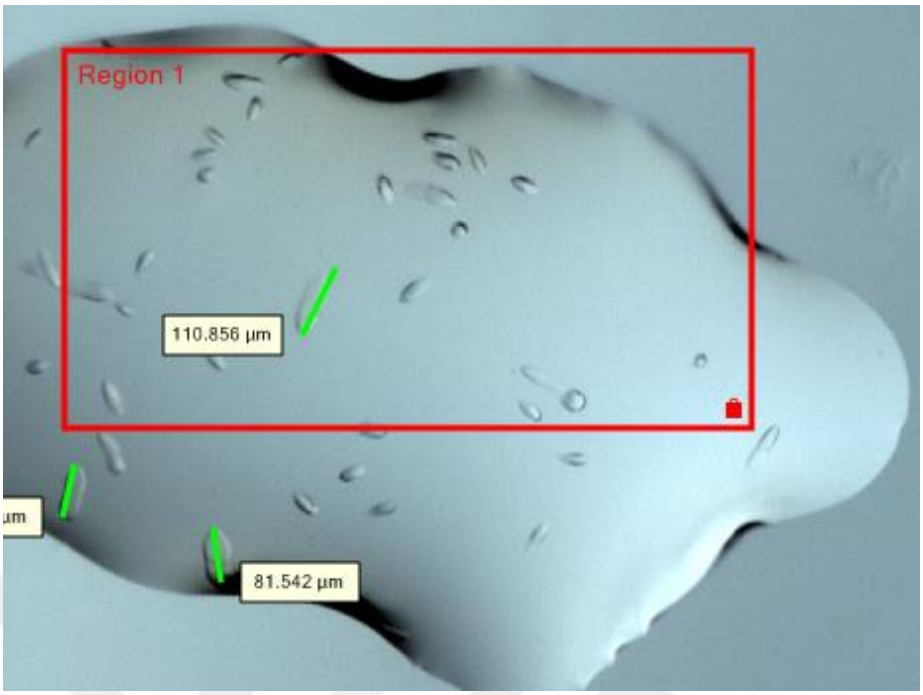


Figure 3.5: Crystals from 5mg/ml ANK T1927-G2126 apo-protein initial screenings. 8mg/ml ANK T1927-G2126 was used for both drops. **A:** The reservoir solution contained 0.1M Imidazole pH:6.5, 1M Sodium Acetate Anhydrous. **B:** 0.1M Tris pH:8.5, 20% PEG1000.

A)



B)

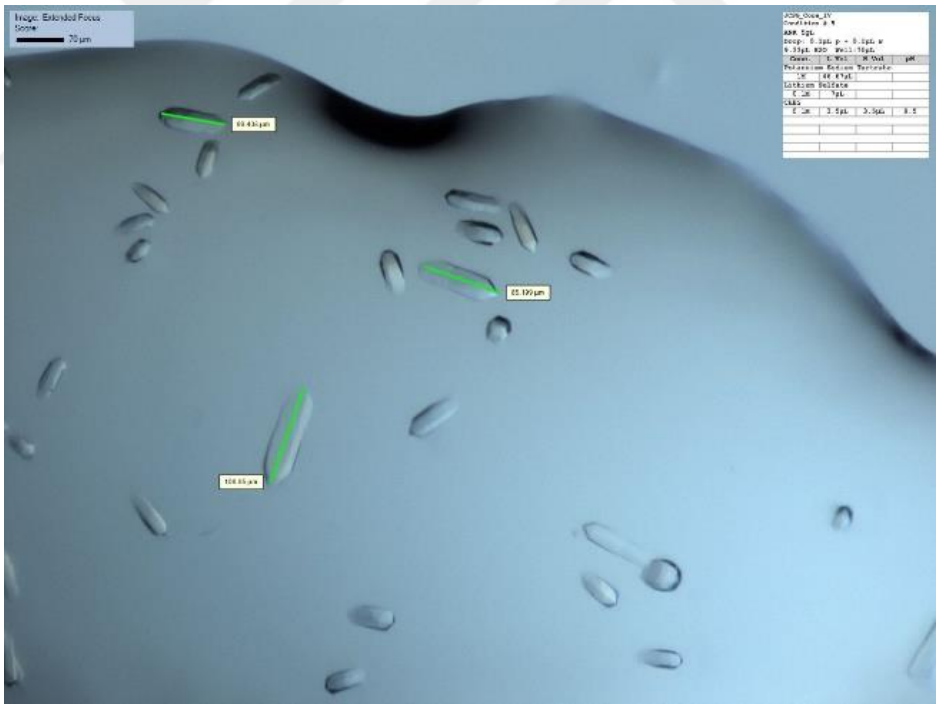


Figure 3.6: Crystals from 5mg/ml ANK E1929-G2126 apo-protein initial screenings. Reservoir solution contain 0.1M CHES pH:9.5, 1M KNaTartrate, 0.1M Lithium Sulfate.

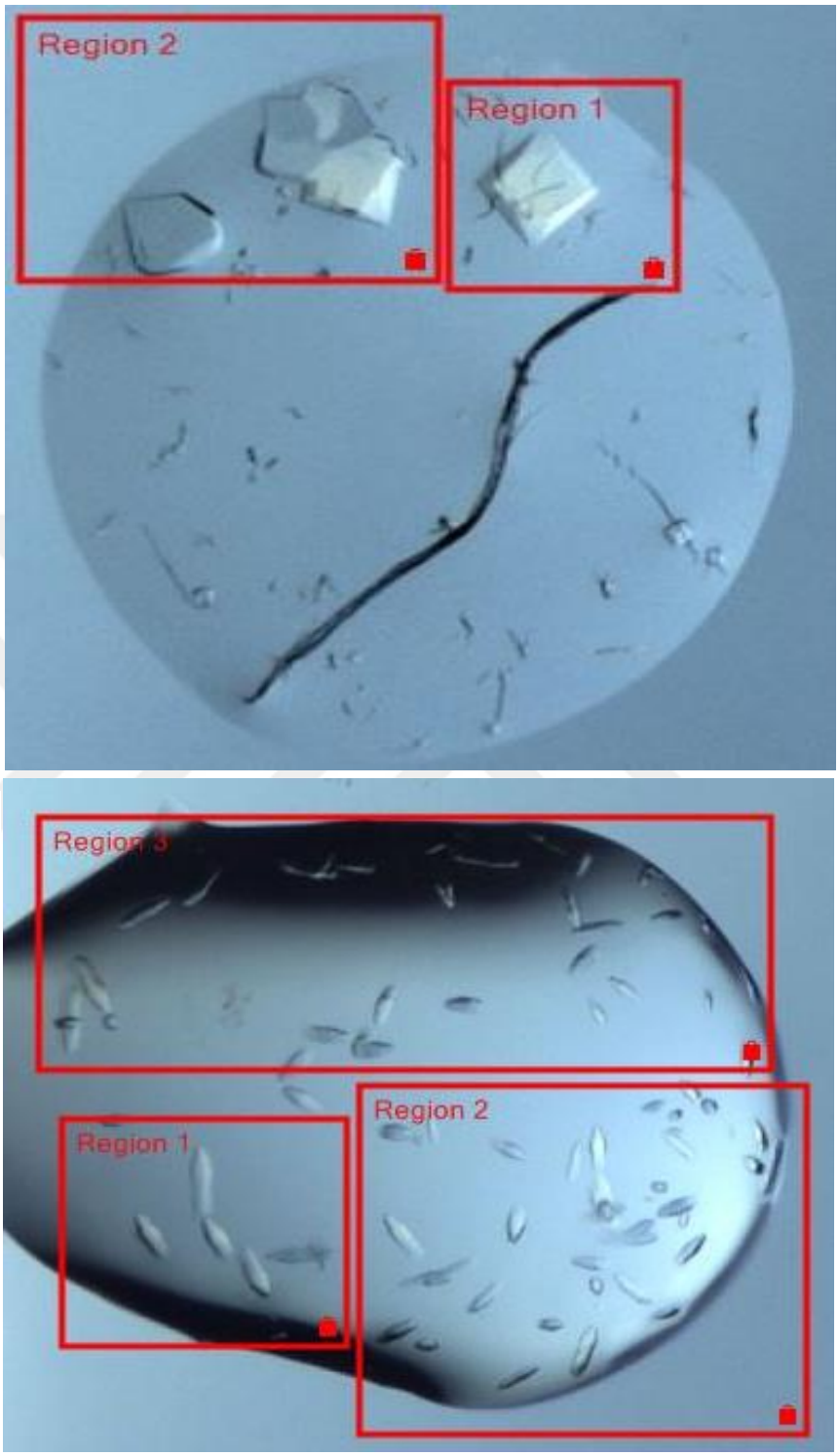


Figure 3.7: Crystals from ANK E1929-G2126 protein mixed with compound I (Table 2.2), initial screenings. **A:** Buffer conditions are 0.1M MES pH:6, PEG6K 20%. Protein and fragment mix ratio is 1:1. **B:** 0.1M CHES pH:9.5, 1M KNaTartrate, 0.1M Lithium Sulfate. Protein and fragment mix ratio is 1:1.

3.2.2. Refinements and Optimization of Crystal Growth Conditions

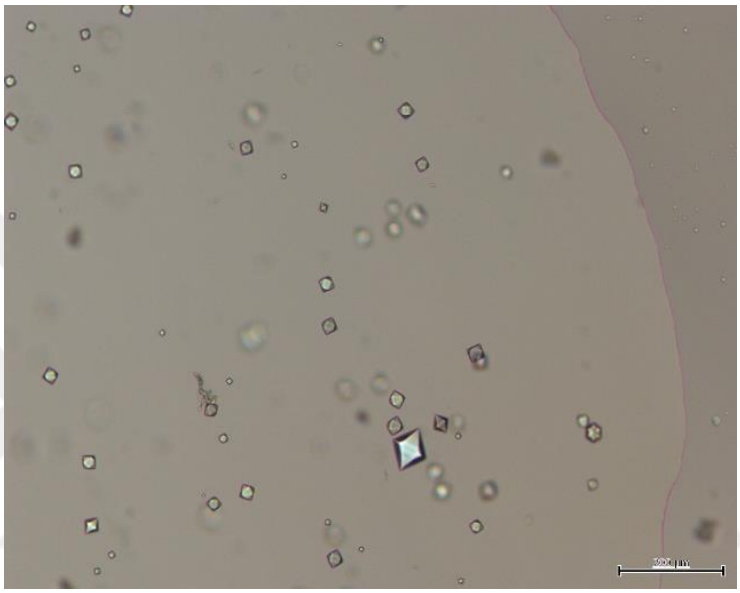
There were 9 crystal conditions from the initial screenings with ANK T1927-G2126 protein fragment that gave crystals and those conditions were further refined by using 24 well plates and relevant buffer conditions in order to optimize and get reproducible crystal refinement conditions (Table 3.1).

Table 3.1: Refinement conditions that ANK T1927-G2126 AND ANK E1929-G2126 protein fragments formed crystals.

NICD-ANK	Protein Amount	Buffer (M/pH)	Salt (M)	Solvent (%)
T1927-G2126	8-5mg/ml	1*M* Tris, pH: 7.5-9.0	NaAcetate 0.2*M*	PEG4K 27-32%
T1927-G2126	10-5mg/ml	1*M* Imidazole pH:6.5-8	NaAcetate 0.7*M*-1.2*M*	
T1927-G2126	5mg/ml	1M Tris pH: 7.5-9.0		PEG1K 17-22%
T1927-G2126 **E1929-G2126**	5mg/ml	1M Imidazole pH: 5.5-7.0	NaAcetate 0.9-1.2*M*	
T1927-G2126 **E1929-G2126**	5mg/ml	0.5M CHES pH: 8-9.5	LiSO4 0.1-0.135*M*	KNaTartrate 1-1.35*M*
T1927-G2126 **E1929-G2126**	8-5mg/ml	1M Tris pH:8.5-9-9.5		PEG1K 20-25-31%
T1927-G2126 **E1929-G2126**	8-5mg/ml		Di-ammonium HPO4 0.1-0.25*M*	PEG3350 18-23%
E1929-G2126	5mg/ml	1M Tris pH: 7.5-9.0	Di-ammonium HPO4 0.7-1.2*M*	
E1929-G2126	5mg/ml	1M Imidazole pH: 5.5-7.0	NaAcetate 0.7-1.2*M*	
E1929-G2126	5mg/ml	0.5M CHES pH: 8.5-10	LiSPO4 0.07-0.12*M*	KNaTartrate 0.7-1.2*M*
E1929-G2126	5mg/ml		Di-ammonium HPO4 0.1-0.25*M*	PEG3350 18-23%

ANK E1929-G2126 protein fragment formed crystals within 8 buffer conditions at 4C degree from the initial screenings. Five of those buffer conditions were further refined and crystal formations were observed with all those conditions repeatedly. There were buffer conditions that both of the protein fragments formed crystals but with different crystal sizes. It turned out that, ANK E1929-G2126, formed bigger protein crystals (Figure3.8) in comparison to ANK T1927-G2126 protein crystals (Figure 3.9), also a faster crystal formation was observed with ANK E1929-G2126 protein during the study.

A)



B)

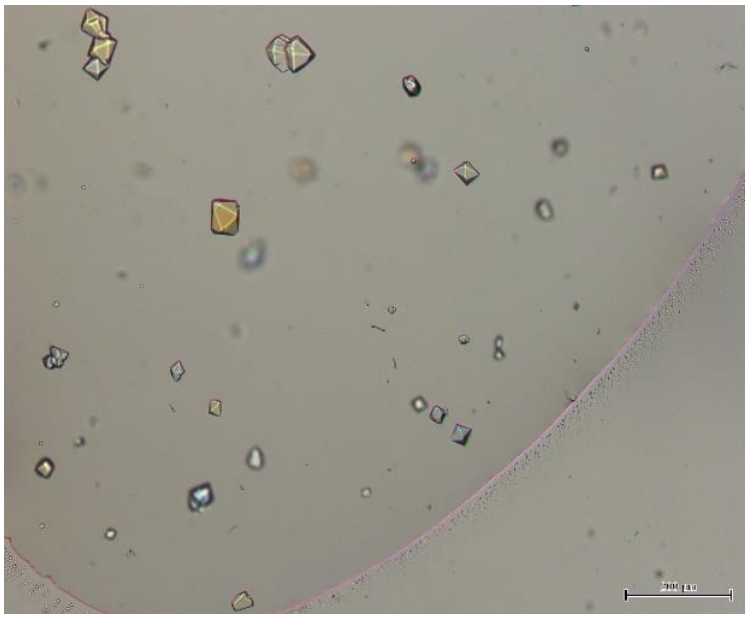
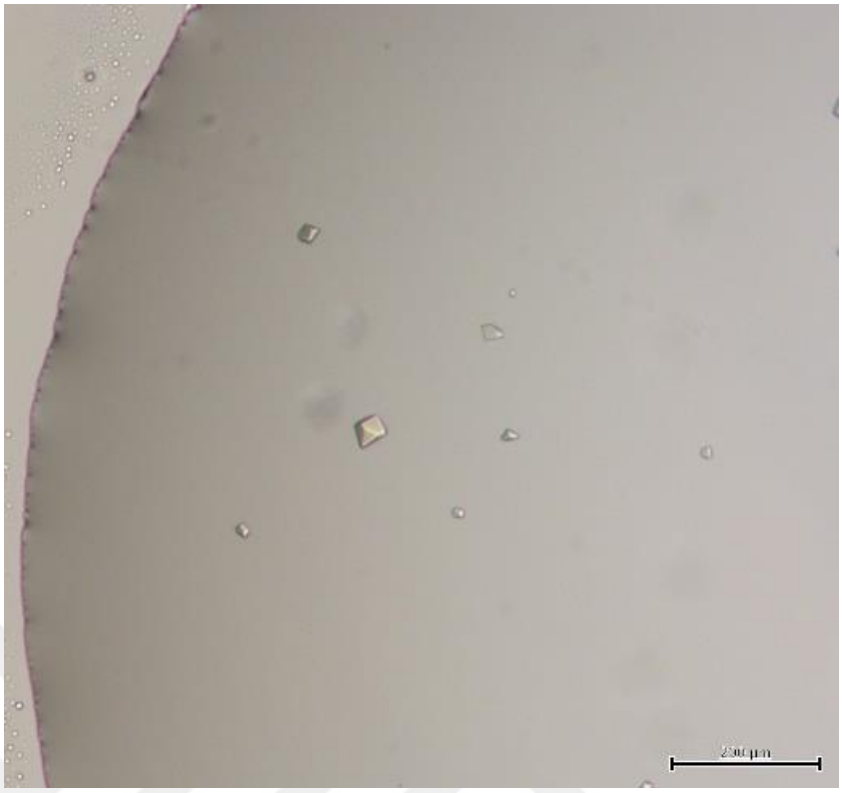


Figure 3.8: ANK E1929-G2126 protein crystals under different buffer conditions. Both crystals formed at 4C degree. **A:** 0.1M CHES pH:10, 0.1M LiSO4, 1.1M KNa Tartrate. **B:** 0.1M Imidazole pH:6.5, 0.07M Na Acetate.

A)



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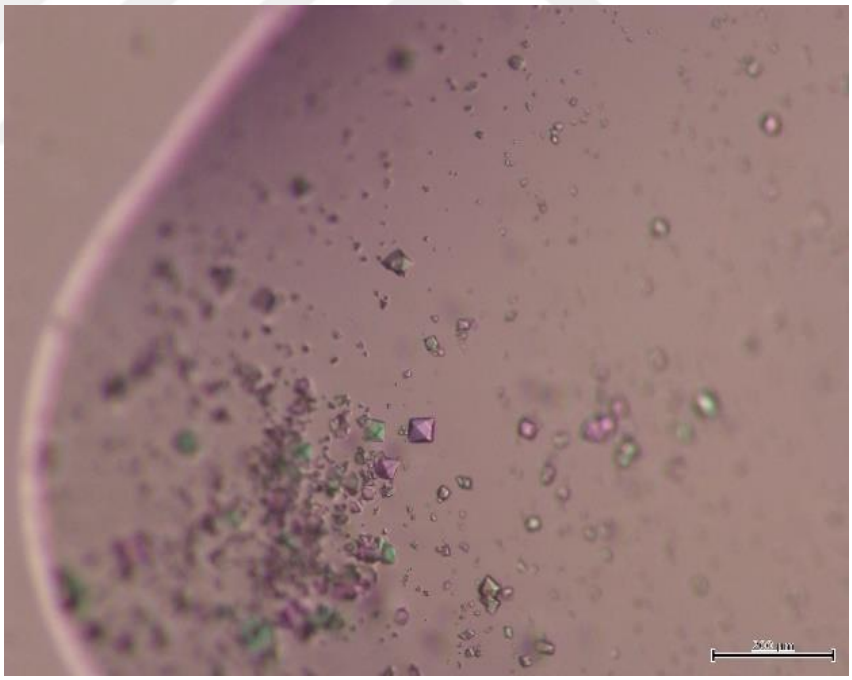
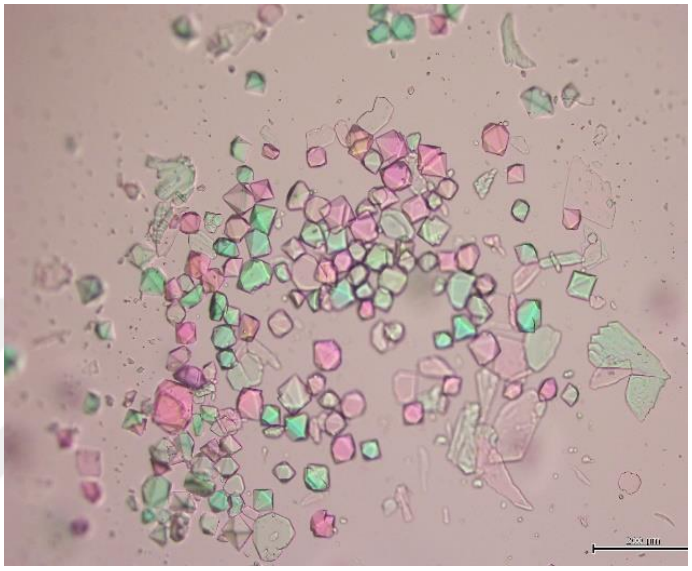


Figure 3.9: ANK T1927-G2126 protein crystals with different buffer conditions. Both crystals were formed at 4C Degree. **A:** 0.1M Imidazole pH: 6.5, 0.07M Na Acetate. **B:** 0.1M Imidazole pH: 8, 1.2M NaAcetate.

3.2.4. Fragment-protein refinements

Buffer conditions from initial screenings were applied and further optimized during the refinement set ups. Almost all of the optimized conditions with apo-ANK E1929-G2126 gave crystals. The compound and the crystals were mixed with the ratios 1:1, 1:2, 1:3 respectively. We mostly got crystals with the 1:1 ratio in initial screenings which applied to the refinements (Figure 3.10). We got protein crystals that were co-crystallized with compound number 9, 2 respectively.

A)



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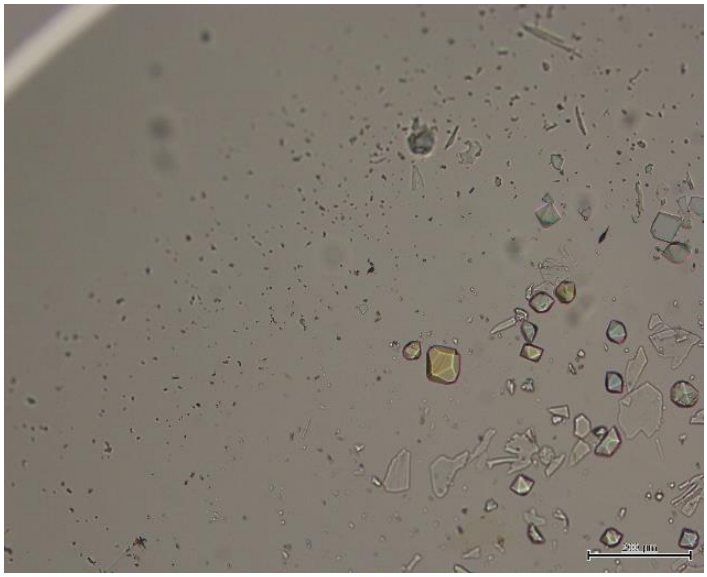
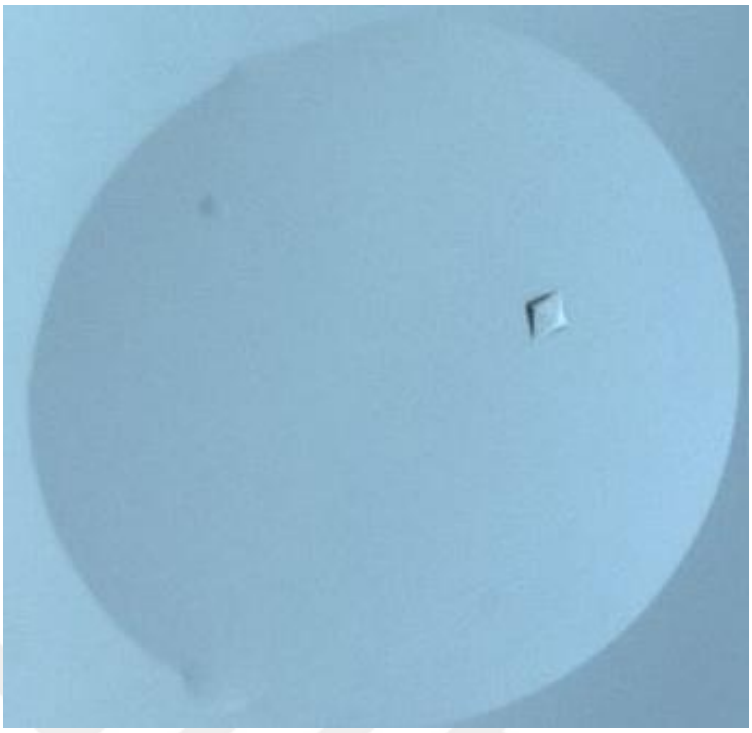


Figure 3.10: ANK E1929-G2126 protein crystals after co-crystallization with compound B (Table 2.2) under different buffer conditions. Both crystals were formed at 4C degree. A: 0.1M CHES pH:8.5, 0.12M LiSO4, 1.2M KNa Tartrate B: 0.1M CHES pH:10, 0.1M LiSO4, 1M KNa Tartrate.

A)



B)

Figure 3.11: ANK E1929-G2126 protein crystals from initial screenings with/without compound I (Table 2.2) with 1% DMSO added buffer conditions. **A:** Crystal formation with ANK E1929-G2126 in 0.1M MES, 20% PEG6K and 1% DMSO. **B:** Crystals of ANK E1929-G2126 with compound I with 0.2M Sodium Sulphate, 20% PEG3350.

3.2.5. X-ray Diffraction and Structure Analysis

ANK E1929-G2126 protein was used with co-crystallization experiments intending the discovery of fragment-based interactions. Crystals of compound I (Table 2.2) were picked from initial screening plates were tested for diffraction. Diffraction data was analyzed via CCP4 and structure refinements were started by using COOT software. The structural data was aligned with previously published PDB data 1yyh, which has structural information for the Ankyrin domain of Notch membrane protein. In our structure data, 6 protein chain were shown with different lengths(Figure 3.12)

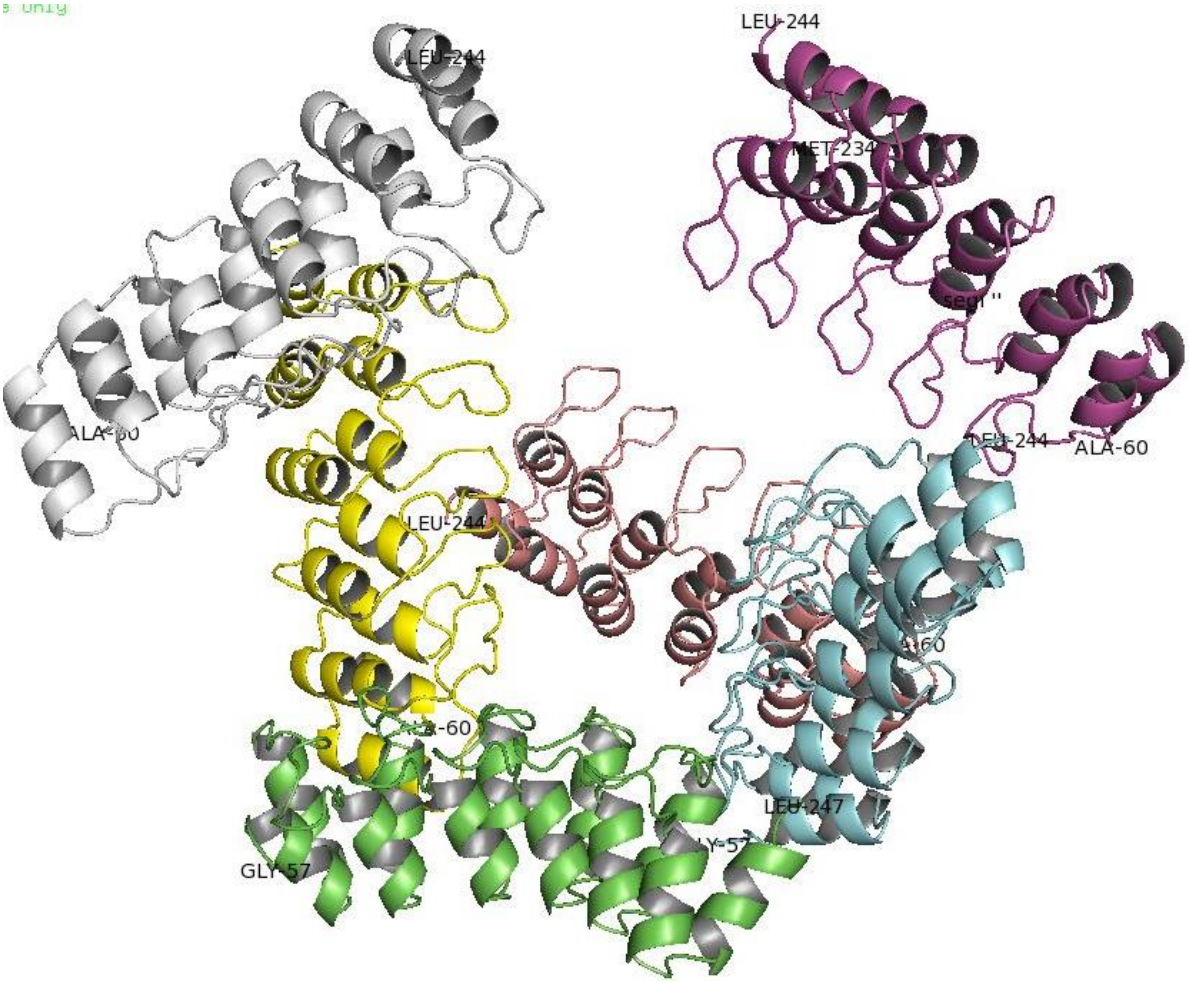


Figure 3.12: ANK E1929-G2126 protein chains from the solved(95%) structural data (images were made by using PyMol).Statistics related data of the structure was collected by using wwPDB validation service (Table 3.2).

The X-Ray resolution of the ANK E1929-G2126 was 2.2 Å. The space group was decided as P 1 21 1 which also means that space group is chiral and acentric. B factor is a quantity that measures the vibrational motion of an atom, which is then a more related feature in comparison to interface size that is used to characterize protein binding (Q. Liu, Li, & Li, 2014). The average B-factor of ANK E1929-G2126 was defined as 33.0 including all 6 protein chains in the crystal structural data.

3D-protein structure was analyzed by PyMOL, since we shortened the Ankyrin protein from its N-Termini, the comparison between the N-termini of ANK E1929-G2126 and previously published 1yyh ANK protein was made in term of the newly formed bonds or the distances between the residues in the protein structure.

Table 3.2: Data Refinement and Statistics

Property	**Value**
Space Group	P 1 21 1
Cell Constants a, b, c, α, β, γ	97.67Å 109.30Å 97.86Å 90.00° 119.32° 90.00°
Resolution(Å)	48.93-2.20
% Data completeness	95.2(48.93-2.20)
R_{merge}/R_{sym}	Not available
Refinement Program	REFMAC 5.8.0155
R, R_{free}	0.210, 0.259
Total number of atoms	9139
Average B, All atoms(A^2)	33.0

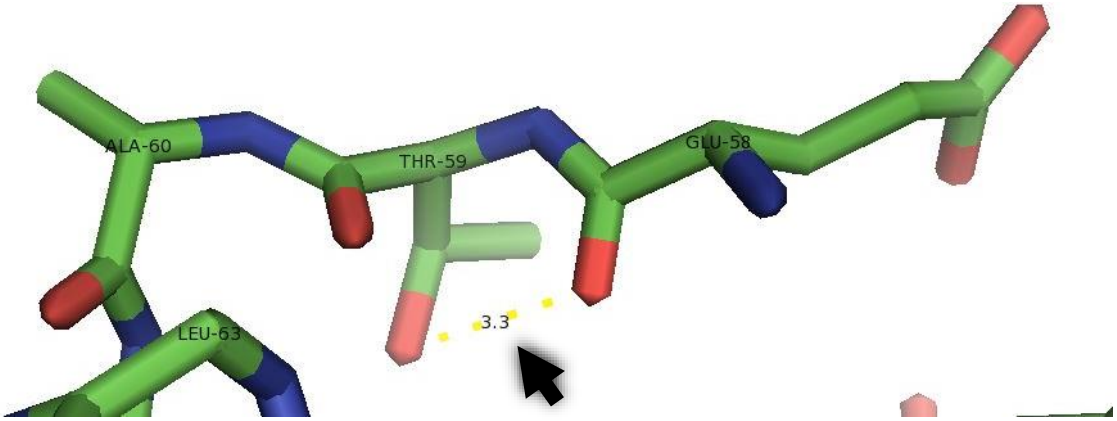


Figure 3.13: The N-termini of Ankyrin protein (E1929 is represented as GLU-58) and newly formed interaction between the residues THR-59(T1930) and GLU-58(E1929).

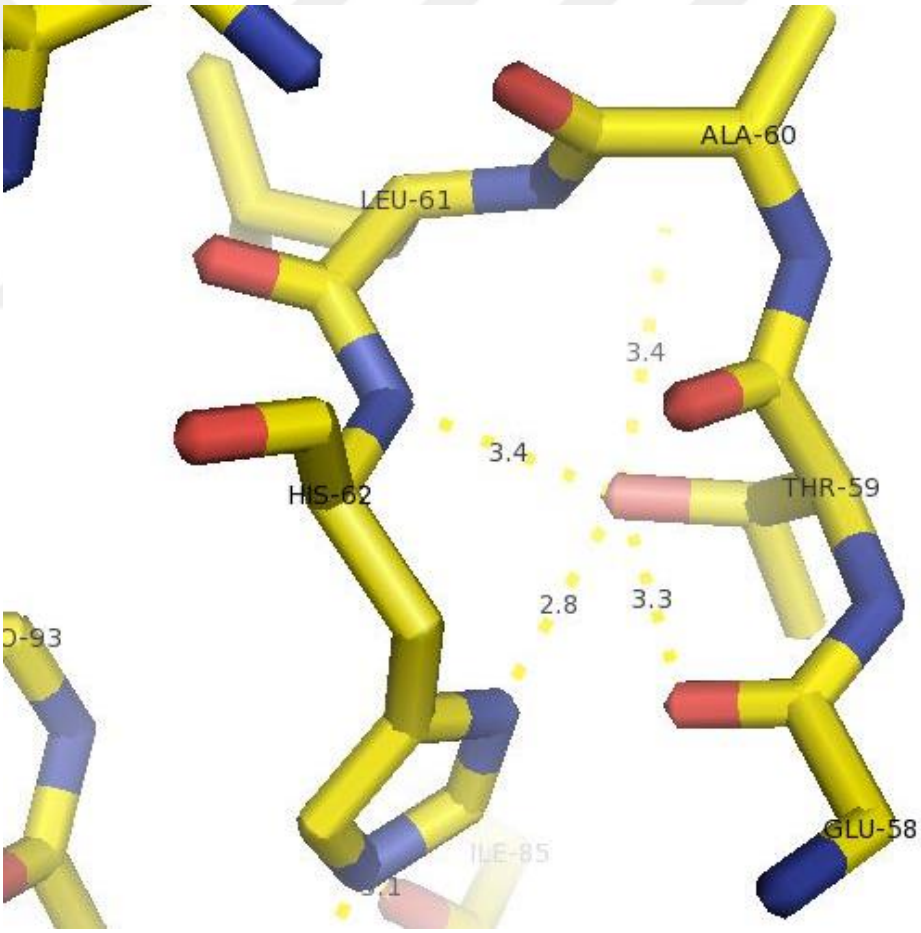


Figure 3.14: H-bonds and the distances between the residues at N-termini of ANK E1929-G2126. Side chains of Threonin-59 (T1930) and Glutamine-58 (E1929) interacts with the amino groups on Hist-62 amino acid (H1933).

Between the ANK E1929-G2126 and 1YYH (PDB) structures, the length of the H-bonds between the residues was slightly different. In ANK E1929-G2126 structure, longer bonds formed except the between threonine and the imidazole ring of histidine amino acid (HIS-62, H1933) which is about 2.8 Å. Also, lack of H-bond between the GLU-58 (E1929) and Imidazole ring of HIS-62 was seen which happens to be present in 1YYH structural data with the bond length of 3.1 Angstrom (Figure 3.14 and Figure 3.15).

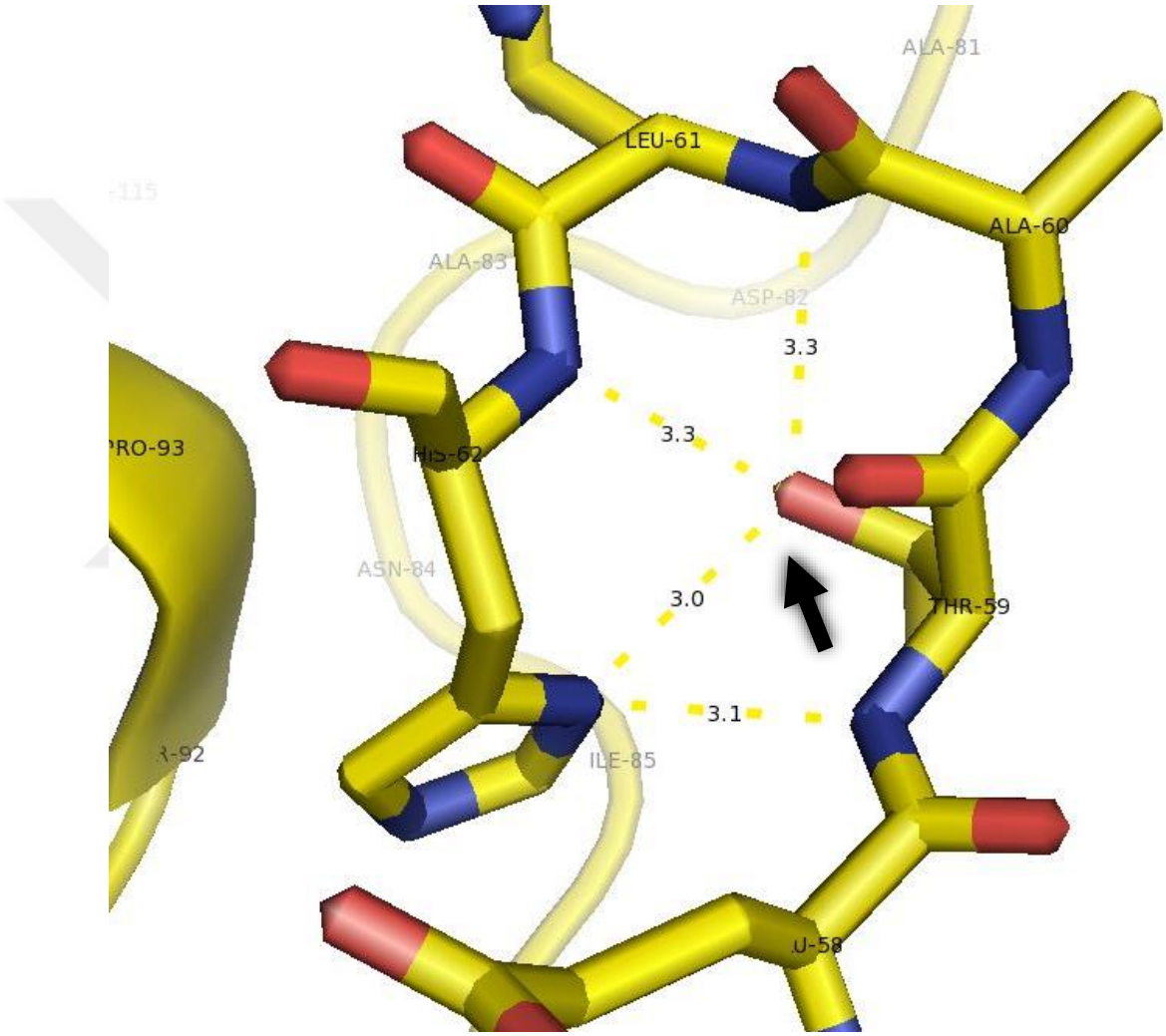


Figure 3.15: H-bonds and the distances between the residues at N-termini of 1YYH(PDB). Side chains and amino-groups of Threonin-59 (T1930) interacts with the amino groups of Leu-61 (L1932) and Hist-62 (H1933). The amino group of Threonin-59 (T1930) interacts with imidazole ring of His-62 (H1933).

4. DISCUSSION

In this study, it was aimed to gain more detailed structural data regarding Ankyrin domain of the Notch1 protein which shows an increased expression in Acute lymphoblastic leukemia(ALL) patients (Weng et al., 2004). Ankyrin domain is very important for Notch Transcription Complex (NTC) assembly since it induces the activation of the complex via activating CSL which in general repress the gene, and with the completion of the complex with MAML-1, the gene transcription starts (Choi et al., 2012).

There were several studies that tried to prevent the assembly of NTC such using antibodies, designing stable like protein structures from MAML-1 (Moellering et al., 2009) which did work but was not so efficient in comparison to the specifically designed drugs that has potential to block binding or change the target proteins confirmation that may then change the function of the protein, even ending up being inactivating it as well as its role in the pathway. That is why we wanted to try fragments which were detected via NMR and tried to see if those fragments showed any interaction with the Ankyrin domain proteins. If we observed any interaction with those fragments, then we could continue with the whole application of fragment-based drug design discovery method, which was not possible in our case since we did not observe any interaction so far.

X-ray crystallography is a major technique in structural biology that gives detailed 3-dimensional information about the proteins in question as well as the interactions happening between two molecules. It is hard to get protein crystals from every protein. Every protein does not form crystals while some of them just form crystals under certain conditions such as buffer content, temperature, pH or incubation duration etc. To be able to get protein crystals, the crystal homogeneity and concentration is very important which is why there are multiple purification steps following the protein expression. In column chromatography, which separates the samples depending on their binding affinity, we first isolated the ANK protein fragments, which was then tagged with GST, from other proteins in the mix. After that, we digested the ANK proteins with TEV protease because the expressed protein had a TEV digestion site between GST tag and ANK protein

sequence. Following the digestion, second Column purification was performed this time GST protein was kept on the column while the ANK protein fragments were collected from the flow through. The collected flow-through then further purified via size exclusion chromatography (also called as gel filtration), in which the proteins were separated by their molecular weight.

We used purified proteins for initial screening setups where we checked crystal growth as well as its growth conditions. It is important to check precipitation of the proteins before every screen set up because once the precipitation starts, it causes further breakdown of the protein and prevents crystal formation. Also, higher protein concentration is important but since we got precipitation after 12mg/ml of concentration and got crystal seeds in the refinement with high concentration, we kept the protein concentration between 5-8mg/ml. It was also seen that apo-proteins formed bigger crystals in buffers that had 1%DMSO in it (data not included) so it may also be useful to add different amounts of DMSO to the decided precipitant conditions to see if there are any significant improvements with the crystal size.

Fragment-Based Drug Design is a technique where if there is an interaction observed, can be a beginning for the building of a potential drug against the disease-related molecules. We got a chemical compound information where chemical compounds were selected via NMR against the Notch1 protein. We used these fragments to check any potential interaction between Ankyrin domain and the fragments while optimizing the crystallization conditions for further studies. We used a co-crystallization technique which we dissolved compounds in 1% DMSO containing buffer solution which was the same buffer from the size exclusion chromatography except for the DMSO, then we prepared saturated compound solution. To increase the solubility of the compounds in the solution, we kept them in a sonic bath at least for 15 minutes. They were mixed with the proteins during initial screening step with the ratios of 1 protein and 1,2,3 from the compounds respectively in which we got crystals with the 1:1 ratio but rarely saw crystal with 1:3 ratio drops. It can be assumed that the fragment caused some changes in protein drop. Although we did not see any changes even on the 35^{th} day, there may be more time needed for crystal formation.

After growing crystals in refinements, there were two different type of Cryoprotectant solutions were used at the crystal fishing process. Cryoprotectants are the solutions that helps to preserve the crystals while preventing the extra water molecules from the structure. We used 30% glycerol with the apoprotein crystals. For the co-crystallization with the compounds, the 100% glycerol was diluted within previously prepared saturated compound solutions just before the fishing with the ratio of 1:2 causing glycerol to be 33%. It is suggested that including the ligand compound in cryo solution also increases the chance of fragment-protein interaction since there is compound present in every step in between refinement to crystal diffraction via X-ray.

Although we got structural data regarding ANK T1927-G2126 (data not included) and ANK E1929-G2126, there were not any compound found anywhere in the structure which we can suggest that those fragments do not interact with Ankyrin domain of the Notch1 protein. For that, there are other three more strategies to obtain crystals of protein-ligand complexes; co-expression of the protein with a ligand of interest, use of the ligands during the protein purification and co-crystallization and soaking (Hassell et al., 2007). When the fragments soaked in the same buffer composition with the crystals it increases the crystal stability and longevity during the soaking process. Fragments usually dissolved better in DMSO which is an organic solvent, with the percentage between 1-20. Although it shows the negative effect of the diffraction, adding DMSO may be useful when added during crystallization process for producing crystals which can be more tolerant to the presence of DMSO during soaking. It may also beneficial to add cryoprotectants in the compound soaking solution to evade any problems during crystal freezing step (Davies & Tickle, 2012).

5. CONCLUSIONS

In this study, we expressed and crystallized two ankyrin protein fragments ANK E1929-G2126 and ANK T1927-G2126 respectively. Ankyrin is one of the domains on a Notch1 protein that carries an important role in the assembly of Notch Transcription Complex.

We optimized several conditions for crystal growth which are reproducible and give well-diffracting crystals for both of the Ankyrin protein fragments (Table 3.1). This optimization will be useful for the further studies regarding the structural studies of the assembly of NTC. We also got structural data of ANK E1929-G2126 with the resolution of 2.2 Angstrom which showed new bond formation on the N-Termini of the protein. It is seen that shortening the N-termini caused changes at h-bond lengths as well as absence and formation of new bonds in the process.

It is also concluded that the crystals with compounds that were selected via NMR do not interact with ANK E2129-G2126 fragment since no interaction was observed in the structural data.

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APPENDICES

Notch Ankyrin Domain Sequence (T1927-G2126)

TGETALHLAARYSRSDAAKRLLEASADANIQDNMGRTPLHAAVSADAQGVFQI
LIRNRATDLDARMHDGTTPLILAARLAVEGMLEDLINSHADVNAVDDLGKSAL
HWAAAVNNVDAAVVLLKNGANKDMQNNREETPLFLAAREGSYETAKVLLDH
FANRDITDHMDRLPRDIAQERMHHDIVRLLDEYNLVRSPQLHG

SDS-PAGE gel and Buffers

Stocking Gel	Separating Gel
Acrylamide (37,5:1) 4 ml	Acrylamide (37,5:1) 0,85 ml
SDS-Stocking Buffer 3,75 ml	SDS-Separating Buffer 2,5 ml
H2O 2,25 ml	H2O 1,6 ml
SDS (20%) 50 µl	SDS (20%) 25 µl
APS 100 µl	APS 100 µl
TEMED 10 µl	TEMED 10 µl

5x Sampling Buffer

10% w/v	SDS
10 *mM*	Dithiothreitol, or beta-mercapto-ethanol
20 % v/v	Glycerol
0.2 M	Tris-HCl, pH 6.8
0.05% w/v	Bromophenol blue

1x Running Buffer

25 mM	Tris-HCl
200 mM	Glycine
0.1% (w/v)	SDS

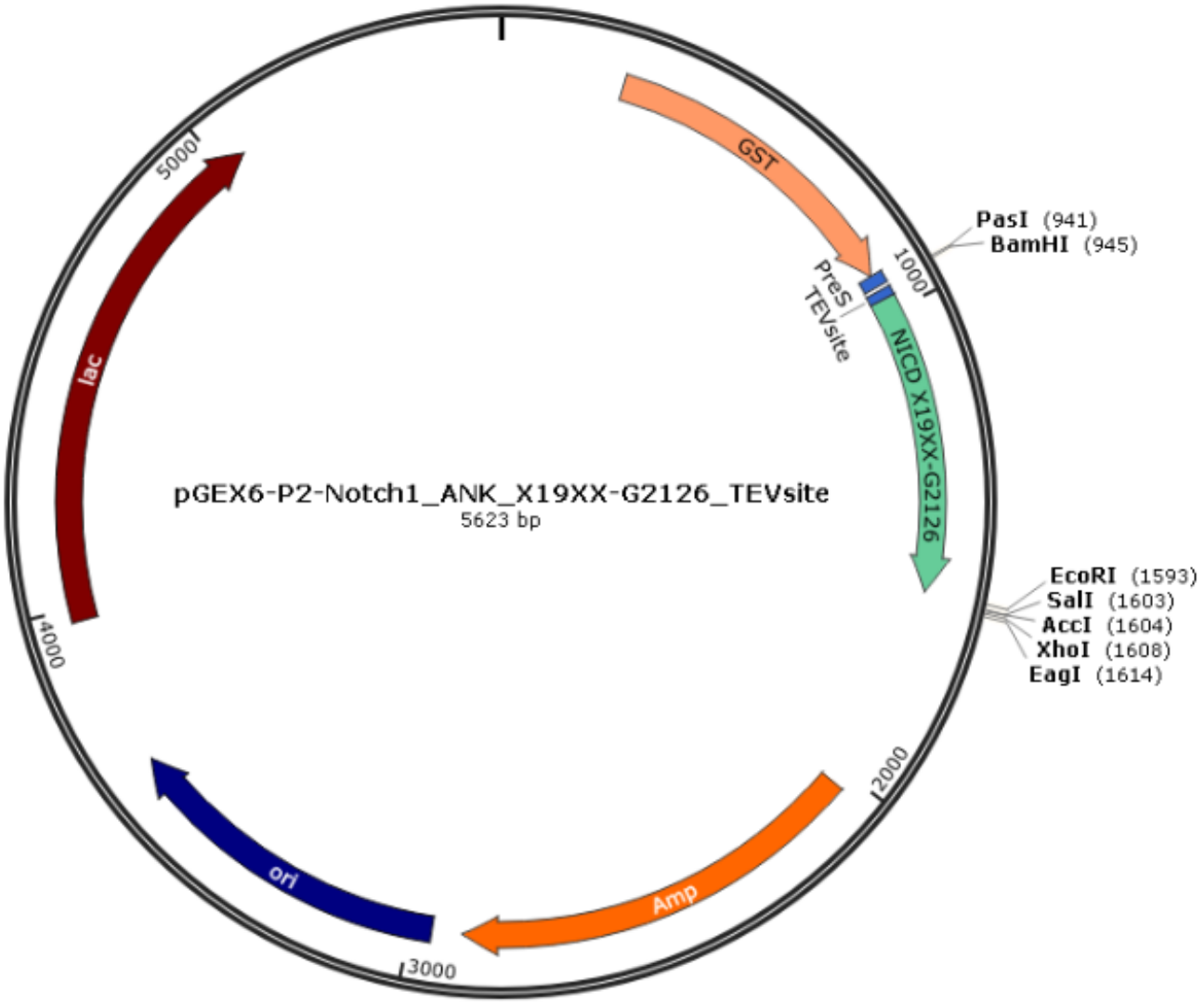
Vector information and Protein sizes.

- pGEX6P-2_GST_NOTCH1_ANK_T1927-G2126_TEVsite(5605bp)

  NOTCH1 ANK T1927-G2126 199aa/ 22.067kDa

- pGEX6P-2_GST_NOTCH1_ANK_E1929-G2126_TEVsite(5599bp)

  NOTCH1 ANK E1929-G2126 197aa/ 21.914kDa



** The Vector information was taken from Sebastian Kiehstaller's M.Sc. thesis: Optimierung der strukturellen Charakterisierung der humanen NOTCH1 Ankyrin Domäne für die Fragment-basierte Inhibitorsuche.2016, Dortmund

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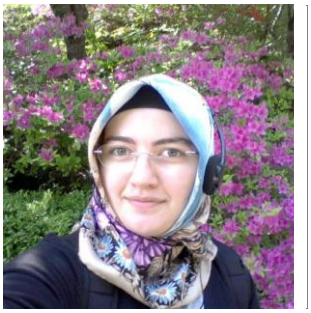

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