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Molecular Mechanisms of Transcriptional Repression by the EWS-FLI1 Oncogene in Ewing's Sarcoma

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Abstract

Ewing's sarcoma is the second most common bone and soft tissue cancer in children with an occurrence of 1-3 cases in 1 million people. Ewing's sarcoma are small round blue cell tumors that express high levels of the cell surface glycoprotein CD99 and carry the t(11;22) (q24;q12) translocation. This generates the EWS-FLI1 fusion gene which can be found in 85% of all Ewing's sarcoma family tumors.

Using Affymetrix gene chip technology, our lab had analyzed the gene expression in six Ewing's sarcoma cell lines and found 73 overexpressed genes and 52 genes repressed by EWS-FLI1. These data indicate that EWS-FLI1 may act not only as transcriptional activator, but also as a transcriptional repressor. Among these repressed genes we found, the Notch ligand JAG1, the Notch target Hey1 and the cell cycle inhibitor p21. It has already been published, that p21 is directly repressed by EWS-FLI1 by binding to a putative Ets motif within the p21 promoter.

p21 is a cyclin-dependent kinase inhibitor, and as a direct target of p53, it arrests the cell cycle in G1 after activation of p53. Several p53 and Ets binding motifs are distributed over 10kb of the p21 5' flanking sequence, but remarkably there are two sites where both, p53 and Ets motifs were found in a cluster. We describe here for the first time a gene reporter analysis of 14 reporter gene constructs which contain overlapping parts covering 10kb of the p21 promoter region. shRNA mediated knock down of EWS-FLI1 resulted in the upregulation of p21 in those two constructs which contain both Ets and p53 binding sites in a p53wt Ewing's sarcoma cell line, but not in a p53mut cell line. Induction of p53 via Etoposide treatment and knock down of EWS-FLI1 resulted in even higher induction of those two constructs in the p53wt cell line, whereas knock down of p53 reduced them. These data indicate strongly that p53 is involved in the gene expression regulation of p21 by EWS-FLI1.

Reporter gene assays using two JAG1 constructs encoding for 1.7kb and 573bp of the JAG1 promoter region resulted in an induction for both constructs upon knock down of EWS-FLI1 in several cell lines. Our data indicate that the regulatory element responsible for the repression of JAG1 by EWS-FLI1 is within the 573bp. Knock down of EWS-FLI1 resulted in an induction of Hey1 in reporter gene assays using two constructs encoding for 3.9kb and 1.7kb of the Hey1 promoter region. Our data suggest that the 3.9kb sequence is necessary for full repression by EWS-FLI1.

We could demonstrate here that EWS-FLI1 acts as a transcriptional repressor, not only for p21, but as well for the Notch pathway components JAG1 and Hey1.

Zusammenfassung

Ewing-Sarkome sind die am zweithäufigsten auftretenden Knochen- und Weichteiltumore bei Kindern. Sie sind klein-rund-blauzellige Tumore, die hohe Spiegel des Zelloberflächen-Glykoproteins CD99 exprimieren und sich durch die t(11;22)(q24;q12) Translokation auszeichnen. Diese führt zum EWS-FLI1 Fusionsgen, welches in 85% aller Tumore der Ewing-Sarkom Familie gefunden werden kann.

Mittels der Affymetrix Gen Chip Technologie analysierte unser Labor die Genexpression von sechs Ewing-Sarkom-Zelllinien und fand 73 überexprimierte Gene und 52 Gene mit verringerter Expression. Diese Daten legen nahe, dass EWS-FLI1 nicht nur als transkriptionaler Aktivator, sondern auch als Repressor funktioniert. Unter den reprimierten Genen fanden wir den Notch-Liganden JAG1, das Notch-Zielgen Hey1 und den Zellzyklus-Inhibitor p21. Die direkte Reprimierung von p21 durch die Bindung von EWS-FLI1 an putative ETS Motive wurde bereits publiziert.

p21 arretiert den Zellzyklus in der G1-Phase nach der Aktivierung von p53. In den 10kb der p21-Promoter-Region sind mehrere p53 und Ets Bindungsmotive zu finden, aber nur an zwei Stellen beide innerhalb weniger Nukleotide. Wir beschreiben hier erstmalig eine Genreporteranalyse von 14 Genreporterkonstrukten, welche in überlappenden Fragmenten 10kb der p21-Promoter-Region abdecken. EWS-FLI1 Knock-down resultierte in erhöhter Expression von p21 in den beiden p53 und Ets enthaltenden Konstrukten, in einer p53wt Ewing-Sarkom-Zelllinie, nicht aber in einer Zelllinie mit mutiertem p53. Induktion von p53 und Knock-down von EWS-FLI1 erhöhte die Expression von p21 noch stärker, während ein Knock-down von p53 p21 verringerte. Diese Daten legen nahe, dass p53 in die Genregulation von p21 durch EWS-FLI1 involviert ist. EWS-FLI1 Knock-down resultierte in einer erhöhten Expression von JAG1 in Reporter Gen Assays mit zwei JAG1-Konstrukten, die 1.7kb bzw. 573bp der JAG1-Promoter-Region enthalten. Unsere Daten lassen vermuten, dass sich das regulatorische Element für die Repression von JAG1 in den 573bp befindet. EWS-FLI1 Knock-down führte zu erhöhter Expression von Hey1 in Reporter Gen Assays mit zwei Hey1-Konstrukten, die 3.9kb bzw. 1.7kb der Hey1-Promoter-Region beinhalten. Unsere Daten deuten darauf hin, dass für die Repression von Hey1 durch EWS-FLI1 die 3.9kb Sequenz notwendig ist.

Wir konnten in dieser Arbeit zeigen, dass EWS-FLI1 als transkriptionaler Repressor, nicht nur p21, sondern auch die Notch-Signalübertragungsweg Komponenten JAG1 und Hey1 reprimiert.

1 Introduction

1.1 Importance of gene regulation in biological processes with an emphasis on transcriptional repression and its mechanisms

The human genome comprises 30.000 to 40.000 genes, most of them encoding for proteins, but thousands of human genes produce non-coding RNAs as their final product [1]. But only a few hundred of genes show constitutive expression in all tissues, the so called housekeeping genes which are essential for the maintenance of the basal cellular functions. Such genes code for example for ribosomal proteins, RNA polymerases, metabolism enzymes and others [2]. Microarrays revealed that in a typical human cell 10.000 to 20.000 genes are expressed all the time, but the profile is different in each cell type. This is due to the synthesis and accumulation of different RNAs, and hence proteins, during the differentiation of each type of cell and the distinct needs of each cell. Cells are as well able to change their expression profile in response to changes in their environment. Such as liver cells trigger the expression of specific proteins, like tyrosine aminotransferase, as a response to glucocorticoid hormones. In contrast fat cells, exposed to glucocorticoid hormones, reduce tyrosine aminotransferase expression levels [3].

These distinct expression profiles are possible because of gene expression regulation, which is possible at every step of generating a mature mRNA from a gene. During transcription, regulation mainly occurs at the step of initiation. RNA processing can be regulated at the stages of modification, splicing, transport or stability. Translation is usually controlled at the stages of initiation and termination [4].

Since the focus in my thesis is on transcriptional repression mechanisms, I will shortly explain transcriptional activation, followed by a more closely focus on transcriptional repression.

The general function of transcriptional activators, which in the majority of cases consist of a DNA binding domain and a transactivation domain, is to attract, position and modify the general transcription factors and RNA polymerase II at the promoter to initiate transcription [3]. Activators like CREB (cAMP response element binding protein), NF-κB or nuclear receptors act directly or via co-activators on the transcription machinery. Another way is via activators that recruit factors that change

the chromatin structure, like histone acetyl transferases, which loosen the chromatin and thereby allow greater accessibility to the underlying DNA [5]. Another mechanism is that the activator induces a conformational change in an inactive RNA polymerase, which is already bound to the promoter, thereby stimulating transcription [6].

Transcriptional repression may be achieved by several mechanisms (Fig.1) (A) If the binding sites for activator and repressor proteins are rather close to each other, or overlapping, these proteins compete for binding to the same regulatory DNA region. (B) Another possibility is, that activator and repressor are both able to bind to the DNA, but the repressor binds to the activation domain of the transcriptional activator thereby inhibiting its function. It is also possible that the repressor binds firmly to the activator, without binding to the DNA. (C) In the third case the repressor interacts in an early stage with the transcription initiation complex and blocks further binding of transcription factors to the complex. Some repressors act also in a later stage of initiation by inhibiting the release of RNA polymerase from the general transcription factor complex. (D) The transcriptional repressor may also attract a chromatin remodeling complex that induces a repressive chromatin structure over the promoter region. Some chromatin remodeling complexes seem to be specialized to restore the repressed nucleosomal state of the promoter, whereas others, which are attracted by activator proteins, increase the accessibility of DNA packaged in nucleosomes. (E) The repressor recruits histone deacetylase to the promoter, leading to tighter packaging of the chromatin thereby decreasing the accessibility of DNA. Another possibility would be the inactivation of a transcriptional activator by heterodimerization [3].

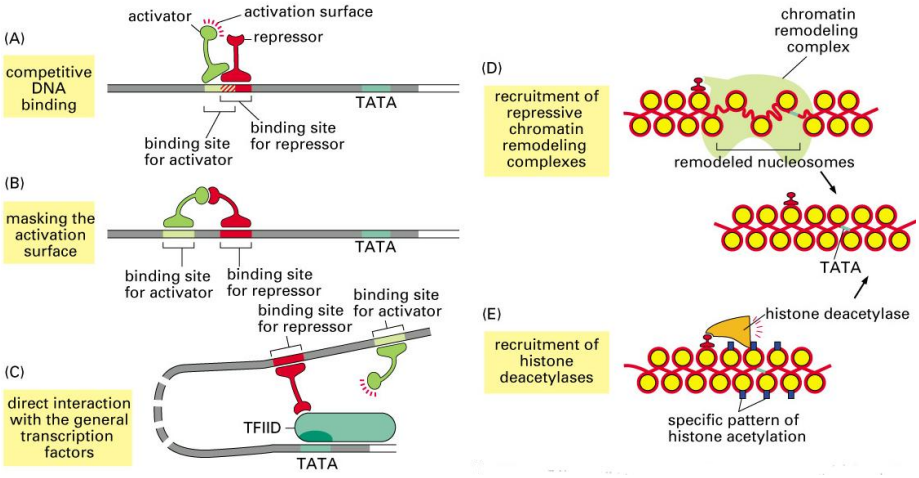

Fig. 1: Transcriptional repression [3]

1.2 Transcriptional repression in disease and cancer

In the following I will give a few examples of diseases in which transcriptional repression is involved.

BRCA1 is a tumor suppressor gene that plays an important role in the repair of DNA damage and surveillance of the cell cycle. After activation of BRCA1 via phosphorylation by the checkpoint kinase ataxia telangiectasia mutant (ATM), BRCA1 interacts with a p53 containing complex that leads to homologous recombination or non-homologous end-joining, which is of great importance in DNA damage repair [7].

BRCA1 is repressed in a large portion of breast cancer patients and is associated with a malignant phenotype [8]. Metastasis associated tumor antigen1 (MTA1) is in contrast up regulated in several cancers. MTA1, a component of the nucleosome remodeling and deacetylating complex, was found to physically interact with an atypical estrogen responsive element on the BRCA1 promoter thereby repressing its expression. Cells with overexpressed MTA1 showed centrosome amplification which has long been a phenotype for BRCA1 repression. Silencing of MTA1 or treatment with a histone deacetylase inhibitor resulted in enhanced levels of BRCA1 supporting the hypothesis that MTA1 represses transcription of BRCA1 [9]. Another study showed that aberrant cytosine methylation, histone hypoacetylation and chromatin condensation act together in the BRCA1 promoter region to repress its expression [8].

The colony-stimulating-factor 1 receptor (CSF1R) is a tyrosine kinase receptor that is essential for macrophage differentiation thereby playing a central role in hematopoiesis. Changes of CSF1R expression is a hallmark of many cancers. In acute myeloid leukemias the t(8;21) translocation product RUNX1-ETO represses CSF1R by binding to the Fms intronic regulatory element (FIRE). RNAi mediated inactivation of RUNX1-ETO resulted in upregulation of CSFR1 expression and macrophage differentiation [10].

The Rett Syndrome is a neurodevelopmental disorder and categorized as a pervasive developmental disorder, like autism, Asperger syndrome and others. It is characterized by loss of language and directed hand movements, as well as ataxia, seizures, deceleration of head circumference and respiratory functions [11]. The

syndom mainly results from mutations in the Methyl-CpG- binding protein 2 (MECP2), belonging to a family of proteins binding to methylated DNA. MECP2 recruits co-repressor complexes and turns chromatin into a repressive state. The brain derived neurotropic factor (BDNF) which plays a role in normal brain development and in learning and memory, is a downstream target of MECP2 and its deregulation in the absence of MECP2 seems to play a role in the neurological phenotype of Rett syndrome patients [12]. ID1, ID2, ID3 and ID4 are as well target genes of MECP2 and encode for inhibitors of differentiation and inhibitors of DNA binding that block transcription factors, which are involved in the regulation of neuronal differentiation genes. The upregulation of the ID genes results from the loss of repression by MECP2 and may explain the arrest in postnatal neuronal maturation in Rett syndrome [13].

1.3 Cancers: brief overview of various types of cancers

There are several classifications of cancer, like the WHO International Classification of Diseases for Oncology, which classifies tumors due to the organ or anatomic location in which they arise [14]. This conflicts with the historical and still widely used histological classification according to the tissue and cell of origin of a cancer type [3]. For example the brain contains connective tissue, as well as lymphoid tissue, therefore melanoma, lymphoma, myeloma, sarcoma and others, may be brain tumors. But the same list would be found in any other site specific classification [15].

Referring to the historical, histological classification the following types of cancers can be found:

Carcinoma derives from epithelial cells and is the cause of 90% of all human cancers [3]. Subtypes of carcinoma are adenocarcinoma, which have a glandular organization and squamous cell carcinoma derived from stratified squamous epithelium [16].

Sarcoma is a neoplasm of the connective tissue formed by proliferation of mesenchymal cells [16, 17] and may arise anywhere in the body. Sarcoma is only 1% of adult cancers, but 15-20% of children's cancers [17].

Lymphoma is a neoplasm of the lymphoid tissue. Lymphoma can be divided into Hodgkin, non-Hodgkin and immunoproliferative small intestinal disease [16].

Leukemia is the malignant proliferation of hemopoietic cells [18]. Leukemia can be divided into acute and chronic leukemia as well as into lymphocytic and myeloid leukemia. In acute leukemia cells do not mature properly and accumulate, whereas in chronic leukemia cells mature, but do not function correctly [16, 17]. Lymphocytic leukemia is characterized by a hyperplasia of the lymphoid tissues and an increased number of malignant lymphocytes and lymphoblasts. In myeloid leukemia the myeloid cell lineage proliferates uncontrolled [16].

Melanoma is a neoplasm of the skin, but since melanocytes derive from the neural crest, it is not a carcinoma. Only 6% of skin cancers are melanoma, but 75% of skin cancer death are due to melanoma [17].

Currently quite an effort is put into the development of new classifications due to molecular markers using microarray data [15, 19, 20].

1.4 Ewing's sarcoma

Ewing's sarcoma is the second most common solid bone and soft tissue cancer in patients less than 20 years [21-23] with an occurrence of 1-3 cases in 1 million people [21, 24] and a 1.5:1/male:female ratio [21, 22, 25]. The frequency is much lower in Asian and African people [22]. James Ewing first described this kind of cancer in 1921 as an endothelioma of the bone, believing that the origin were blood vessels of bone tissue [26]. Nowadays it is assumed that it is of mesenchymal stem cell origin [27, 28].

Ewing's sarcoma belongs to the Ewing's sarcoma family, which contains beside itself, the peripheral primitive neuroectodermal tumors and Askin tumors [21, 24]. Ewing's sarcoma and PNET express high levels of the cell surface glycoprotein CD99 [29] and carry the t(11;22) (q24;q12) translocation which generates the Ews-Fli fusion gene which can be found in 85% of all Ewing's sarcoma family tumors [23]. The difference has been defined so far, that PNET demonstrates neuroectodermal features, while Ewing sarcoma does not [30].

Ewing's Sarcoma is a small round blue cell tumor [21-23, 25] like neuroblastoma, lymphoblastic lymphoma, rhabdomyosarcoma, with a high nuclear to cytoplasm ratio. Typically those cells have scant, faintly eosinophilic cytoplasm that contains glycogen appearing as periodic, acid-Schiff-positive diastase-digestible granules. As well as

indistinct cytoplasmic borders and round nuclei with evenly distributed chromatin and little mitotic activity [21, 22, 30].

The histological differentiation between the Ewing's Sarcoma family of tumors and the mentioned above small round blue cell tumors is according to various markers. Ewing's sarcoma and lymphoblastic lymphoma express CD99, whereas only lymphoblastic lymphoma expresses CD45. Both Ewing's sarcoma and Neuroblastoma express neural specific enolase and S-100, but Neuroblastoma is vimentin negative and neurofilament positive, unlike Ewing's sarcoma. Alveolar rhabdomyosarcoma may express CD99, but as well desmin, myogenin and MyoD1, unlike Ewing's sarcoma. The most difficult differentiation is between Ewing's sarcoma and poorly differentiated small synovial sarcoma, which may express CD99 but lack other histological markers [21]. Further methods like fluorescence in situ hybridization or polymerase chain reaction are required for a definite diagnosis [22].

Ewing's sarcomas mainly arise in bones, like the pelvic bones, the long bones of the lower extremities, bones of the chest walls, ribs and humerus. Primary metastasis is observed in 25% of patients and arises for the most part in bones, bone marrow or lungs [22]. In 15% the primary tumor site is within soft tissue [24].

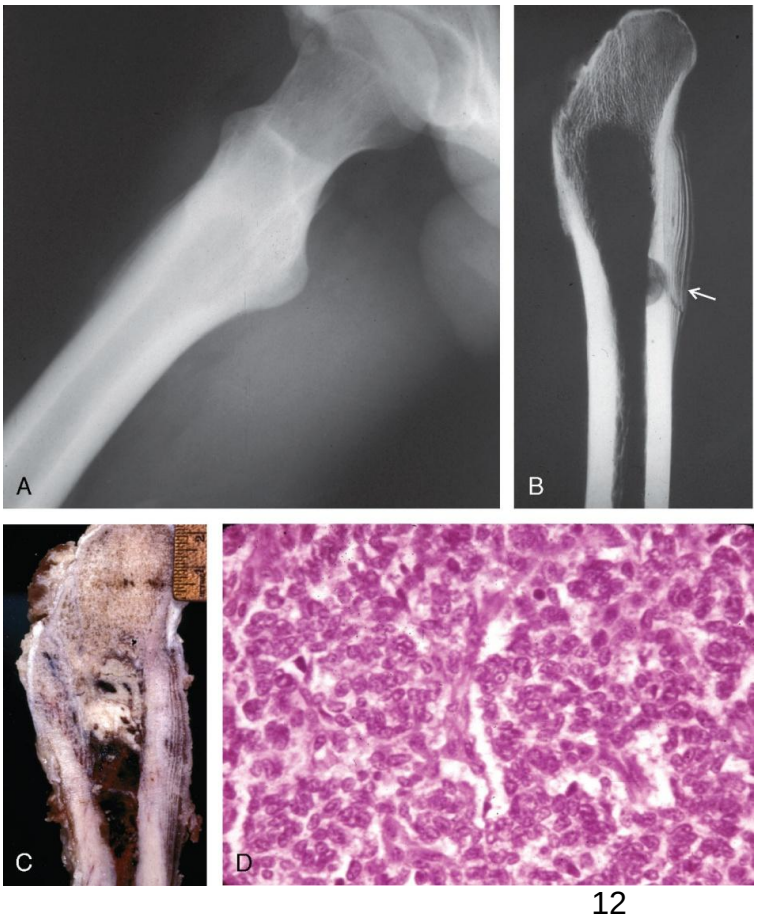

Fig. 2: 15-year-old boy with Ewing sarcoma lesion of the proximal right femur [31].

A: Radiograph of the proximal femur: mild mixed sclerosis with aggressive "onion skin" type periosteal reaction.
B: Radiograph of the hemisected proximal femur.
White arrow: biopsy site
C: Resected gross pathology specimen: lesion with aggressive periosteal reaction.
D: Photomicrograph: Typical features of Ewing sarcoma. [31]

Treatment of Ewing's sarcoma always involves several approaches like systemic chemotherapy, surgery and radiotherapy. After an initial chemotherapy a local treatment of the tumor by either surgery or radiotherapy is performed. Surgery depends on the localization of the tumor, if it is removable, and the age of the patient. This is followed by a second systemic therapy for treatment of microscopic residual disease [31]. In the past when chemotherapeutics were not administered fewer than 10% Ewing's sarcoma patients survived [22], now using the multimodal therapy approximately 50% survive at five years. Patients with localized disease have a much better prognosis with >60% long term survival, while patients who present metastasis can be cured in less than 25% of cases. [21].

1.5 EWS-FLI1

The main characteristic of Ewing's Sarcoma is a translocation leading to a fusion gene between EWS and a member of the ETS family of transcription factors. In 85% of Ewing's Sarcoma the t(11;22) (q24;q12) translocation generating the EWS-FLI1 fusion gene is present [21-24]. EWS encodes a 656 amino acid protein containing a glutamine, threonine and proline rich amino terminal domain, with 40% homology to CTD-polII, three arginine, glycin and proline rich domains and most interestingly a central RNA binding domain [32].

FLI-1 (Friend leukemia integration 1 transcription factor) contains two Ets domains in the 5' and the 3' portion. Together the 5' conserved and the FLI-1 domain constitute the amino terminal transcriptional activation domain. The 3' region contains an 89-amino acid carboxyterminal domain contributing to the transcriptional activation domain and a DNA binding domain [21].

The translocation leads to the fusion of the N-terminal region of EWS and the C-terminal region of FLI-1, resulting in the fusion protein EWS-FLI1 containing the FLI-1 ETS DNA binding domain and the N-terminal transactivation domain of EWS [33].

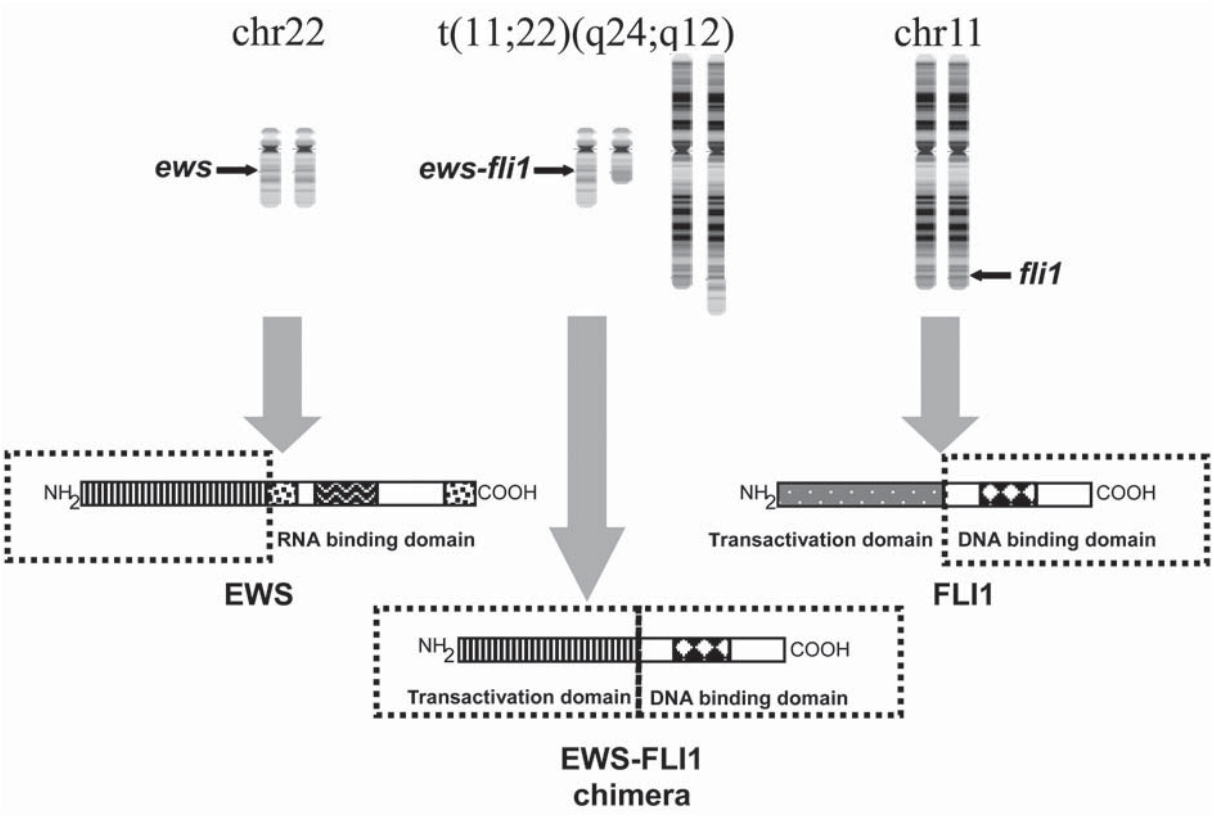

Fig. 3: Translocation between Chromosome 11 and 22 leads to EWS-FLI1 fusion gene [22]

The second most common translocation in Ewing's Sarcoma is t(21;22)(q22;q12) leading to the fusion product EWS-ERG (~10%). Other translocations involving EWS and an ETS gene are even less frequent like, EWS-ETV1, EWS-E1AF and EWS-FEV [21-24, 33].

The oncogenic effect of EWS-FLI1 was revealed by transduction into NIH3T3 resulting in the formation of colonies in soft agar and foci in cell culture [34] and the development of tumors in nude mice [35]. Deletion of EWS-FLI1, EWS or FLI-1 resulted in the loss of the oncogenic effect, indicating that both regions are obligatory for this effect [33, 34].

EWS-ETS fusion proteins are in general transcription factors containing a highly potent transactivation domain and an ETS DNA binding domain. Due to this fact it is supposed that EWS-ETS fusion proteins act as transcriptional activators and lead in so doing to the development of Ewing's Sarcoma, but EWS-ETS may also act as transcriptional repressors [24].

One of the transcriptionally activated target genes of EWS-FLI1 is the platelet derived growth factor C. It was revealed that PDGF-C is expressed in more than 60% of

Ewing tumors and it could be activated in murine NIH3T3 cells by EWS-ETS fusion proteins [36]. Another up regulated factor for cell proliferation is the transcription factor c-Myc, which was found to be highly expressed in several Ewing's Sarcoma cell lines [37, 38] and to be induced by EWS-FLI1 fusion proteins in pMTEF and NGP cells [38]. Id2, inhibitor of DNA binding 2, is as well up regulated in Ewing's Sarcoma cell lines and a direct interaction with EWS-FLI1 could be shown via chromatin immunoprecipitation [37, 39]. Since Id2 is an inhibitor of differentiation of cells, or more precise it inhibits lineage specific genes, this might explain the primitive morphological features of Ewing tumors [39]. Like c-Myc, CCND1 is over expressed in many Ewing's Sarcoma and both activate CDK4 [24, 40], which is essential for the initiation of the cell cycle [24]. Cyclin D1 overexpression is predominantly associated with human tumor genesis leading to deregulated cell growth and proliferation [41]. But not only cell cycle regulation is deregulated, another common alteration in human cancers could be found; telomerase activity and increased expression of telomerase reverse transcriptase was revealed in two Ewing's Sarcoma cell lines and in NIH3T3 cells transformed by EWS/E1AF and EWS/FLI1 [42].

Since the focus in my thesis is on transcriptional repression, I will discuss this topic in the following section in more detail:

1.6 Overview of transcriptional repression targets

Until now the best studied transcriptional repression target for EWS-FLI1 is the transforming growth factor-β type II receptor gene (TGF-βIIR). TGF-β in general is a growth inhibitory cytokine and is responsible for the control of migration, adhesion, differentiation, modification of the microenvironment, tissue growth and morphogenesis [43].

The TGF-β signaling pathway is induced after binding of TGF- β to the type II receptor, which recruits and phosphorylates the type I receptor. The type I receptor phosphorylates receptor associated SMAD2/3, which form dimers or trimers with SMAD4. This complex goes to the nucleus and interacts with DNA- binding cofactors and co-activators or co-repressors to modulate gene expression of TGF-β target genes, like c-Myc, Id2 and the cyclin dependent kinase inhibitors CDKN1A, CDKN2B. Therefore repression of TGF-βIIR leads to a repression of CDKN1A, CDKN2B and an induction of Id2 and c-Myc, which favor uncontrolled cell proliferation [43].

It has been shown that mRNA and protein TGF-βIIR levels were reduced or even undetectable in embryonic stem cells in the presence of EWS-FLI1. Transduction of FLI1 alone induces TGF-βIIR promoter activity as well as antisense RNA to EWS-FLI1 transfected into Ewing's sarcoma cell lines restored TGF- βIIR expression [44, 45]. Additionally it was revealed that also the less common translocations EWS-ERG and EWS-ETV1 lead, when transfected into NIH-3T3 cells, to decreased mRNA and protein levels of TGF-βIIR [46].

Microarrays of siRNA against EWS-FLI1 transfected Ewing's Sarcoma cell line A673 revealed a major regulator of cell proliferation and apoptosis, namely insulin-like growth factor binding protein 3 gene, to be a target of EWS-FLI1 [47].

IGFBP3 is a component of the IGF pathway consisting of cell surface receptors IGF-IR and IGF-IIR, the ligands IGF-I and IGF-II and the IGF binding proteins. IGF-IR is a tyrosine kinase receptor that binds both ligands, which are regulated by the IGFBPs, leading to an activation of the kinase resulting in signaling through cellular pathways that stimulate proliferation and inhibits apoptosis [48].

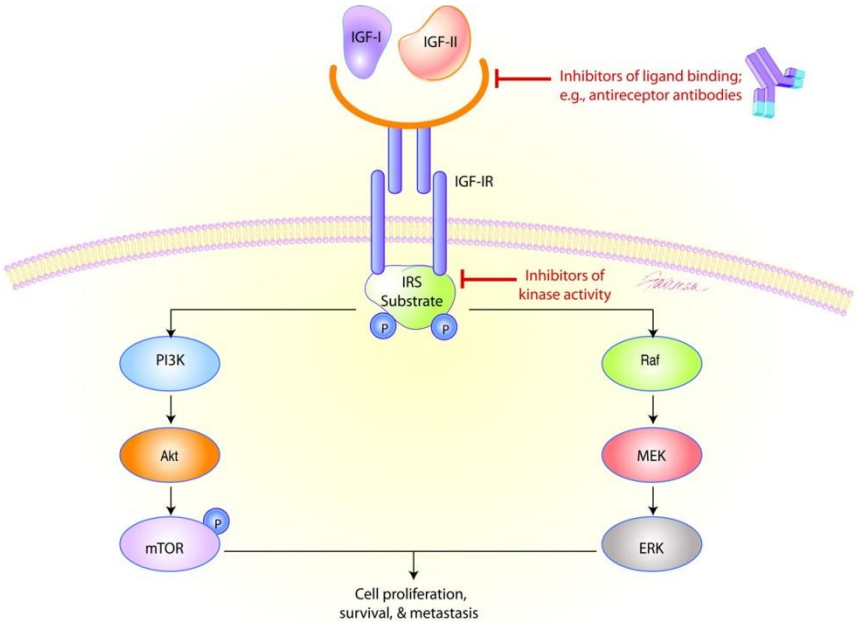

Fig. 4: IGF pathway. ERK: extracellular signal-related kinase, IRS: insulin receptor substrate, MAPK: mitogen-acticated protein kinase, MEK: MAPK-ERK kinase, PI3K: phosphatidylinositol 3' kinase, TOR: target of rapamycin [48].

Knockdown of EWS-FLI1 via siRNA showed an increase of IGFBP3 in microarrays and RT-PCR, overexpression of EWS-FLI1 in HeLa cells resulted in repression of IGFBP3. Luciferase gene reporter assays and Chromatin IPs confirmed this data,

suggesting that IGFBP3 is a direct repression target of EWS-FLI1 [47]. It has been reported several times, that IGFBP3 inhibits IGF-1 and thereby the anti-apoptotic function of the IGF pathway [49]. Repression of IGFBP3 in Ewing tumors may therefore lead to the suppression of apoptosis, which is one of the hallmarks of cancer [50].

1.6.1 p21, p53:

p53 is probably the best studied tumor suppressor and is essential for cell cycle arrest, DNA repair and apoptosis [51-54]. As a transcription factor, p53 induces the expression of p21, cyclinG, Bax, GADD45 and Mdm2 [55]. Under normal circumstances p53 is functionally inactive due to the degradation by the ubiquitin ligase Mdm2. DNA damage, cell stress or oncogenic signaling leads to the stop of ubiquitylation and p53 accumulation [52, 53, 55]. The activation of p53 upon DNA damage is due to several kinases, namely ATM, ATR, Chk1 and Chk2 which phoyphorylate serine and threonine residues, and may acetylate, methylate, ubiquitylate or sumoylate lysines at the carboxy terminal domain of p53. These alterations result in an increase of the half live of p53 in the cell and the ability to bind to specific sequences and therefore promote transcription of genes regulated by this sequences, is improved [56]. The tumor suppressor p14ARF activates p53 upon oncogenic signaling and interacts with Mdm2 inhibiting p53 degradation [52, 53]. Recently it has been revealed that the DNA damage response of p53 is of minor importance for cancer protection than the oncogenic signaling response via ARF [53].

After its activation p53 binds to p53 responsive elements thereby activating transcription of target genes, which initiate either DNA repair, cell cycle arrest, or apoptosis [56]. For example G1 arrest is induced via binding of activated p53 to response elements in p21 promoter region, this leads to elevated transcription of p21 which is a cyclin dependent kinase (cdk) inhibitor and inactivates G1/S phase type cdk and S phase type cdk, holding the cell cycle in G1 [3].

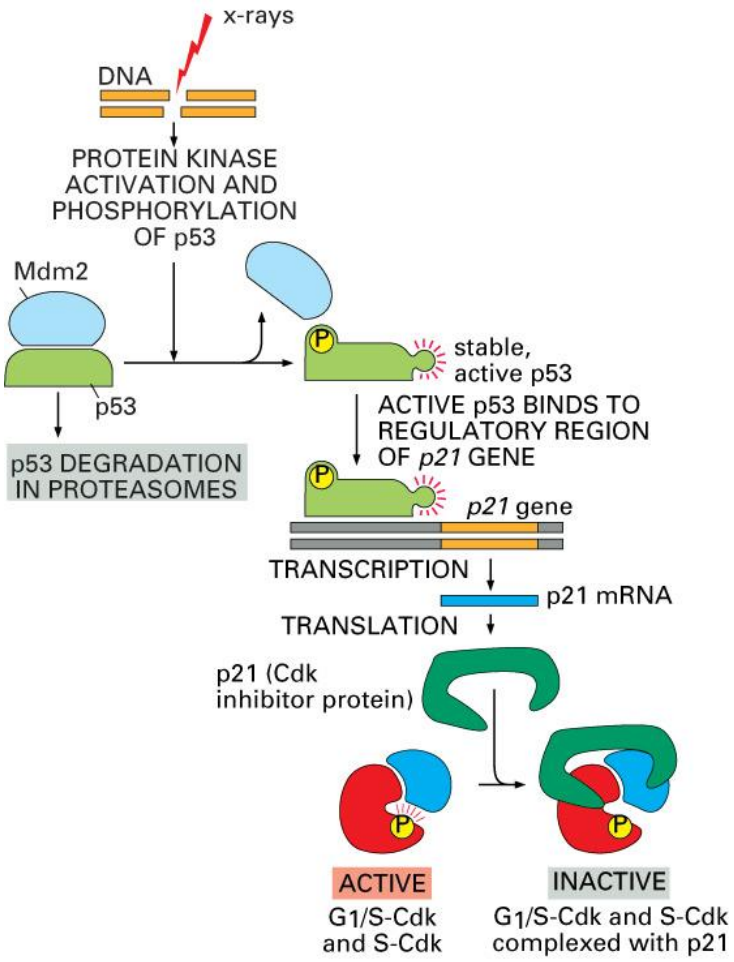

Figure 17–33. Molecular Biology of the Cell, 4th Edition.

Fig. 5: DNA damage induced cell cycle arrest [3]

The initial step for intrinsic apoptosis is the expression of genes, directly regulated by activated p53, like Bax, noxa, puma which enhance the release of cytochrome c into the cytoplasm from mitochondria. APAF-1, as well a p53 regulated gene, interacts with cytochrome c and initiates a protease cascade activating procaspase 9 and thereby caspase 3 resulting in apoptosis [56]. The extrinsic apoptotic pathway results in activation of procaspase 8 and caspase 3 via binding of transmembrane death receptors (Fas, TNF receptor, TRAIL receptor) with the corresponding ligands (FasL, TNF, TRAIL) [57]. p53 mediated senescence is induced when activated Ras oncogene is present in a normal cell [58].

p53 is mutated or truncated in 50% of all cancers [53], whereas it is in only 10% of Ewing's sarcoma but this subset of patients has a noticeable poor outcome [59, 60]. Treatment of Ewing's sarcoma cell lines with ionizing irradiation shows mainly normal DNA damage signal integration, like p53-induced cell cycle arrest and apoptosis [61].

As mentioned above p21/Waf1 is a cyclin-dependent kinase inhibitor and a member of the Cip/Kip family of cdk inhibitors [62]. p21 is a direct target of p53 and arrests the cell cycle in G1 after DNA damage induced activation of p53 [62, 63]. Another mechanism to induce cell cycle arrest is by inhibition of proliferating cell nuclear antigen, a processivity factor for DNA polymerase δ thereby blocking DNA synthesis [64]. In contrary to its inhibitor function, p21 can also stabilize interactions between cdk4/cdk6 and D-cyclins in that way forming active complexes. This only is true for low or intermediate concentrations, while at high concentrations it acts as an inhibitor [62, 64]. It was revealed that p21 in keratinocytes is directly induced by the C-promoter binding factor 1 (CBF1) via Notch1 activation [65].

Several putative Transcription factor binding sites for p53 are located in the p21 promoter region, additionally to the well described sites at -2,27kb and -1,38kb [66], there has recently been found another one at -4,5kb [67] as well as our in silico analysis revealed a number of new potential TFBS for p53. Ets transcription factor binding sites were located at -2,2kb and -1,3kb close to p53 TFBS [68]. Two further Ets motifs and three CBF1 motifs have been identified due to our in silico analysis. Furthermore six Sp1 binding sites are located between the start site and -119bp. STAT binding sites are at -690, -2590 and -4233bp at the p21 promoter [64].

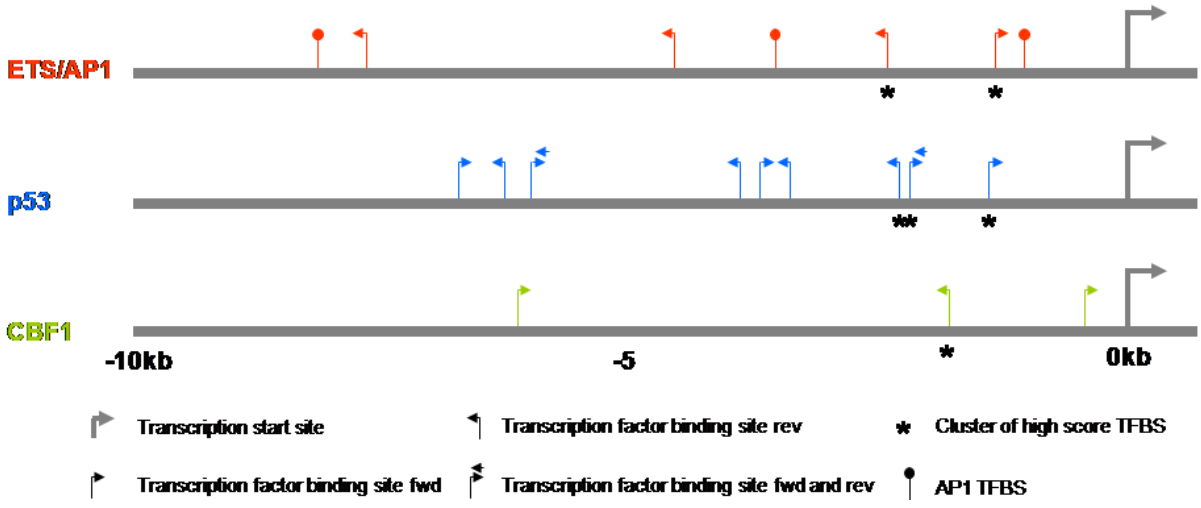

Fig. 6: Transcription factor binding motifs in 10kb of p21 promoter region

Due to the major role of p21 in cell cycle regulation it is not surprising that a number of oncogenes repress p21, but astonishingly mutations of p21 in cancers are extremely rare [62, 69]. Several mechanisms of p21 regulation have been discovered so far, but still the most important one seems to be activation by p53. For example phospholipase D1 or the hepatitis C virus core protein decrease p53 levels thereby

repressing p21. Polo like kinase 1, a M phase cell cycle regulator, or HPV type E6 directly bind p53 and lead either to inactivation or degradation. ΔNp63, a splice variant of the p53 family member p63, competes with p53 for DNA binding on the p21 promoter. c-jun may either repress the p53 promoter directly or interferes with Sp1, another positive regulator of p21, and thereby leads to p21 repression [62].

c-Myc is supposed to negatively regulate p21 via interactions of c-Myc and Sp1/Sp3 [70] as well as via interaction of c-Myc with the initiator binding ZN-finger transcription factor Miz-1 associating directly with the promoter [71].

In some cases epigenetic silencing has been revealed to be another mechanism to repress p21. In Rhabdomyosarcoma tumors the STAT transcription factor binding site on the p21 promoter was found to be methylated, as well as CpG islands near the Sp1/Sp3 sites in lung cancer cell line H719. Hypermethylation of the p21 promoter in bone marrow cells was found in 41% of lymphoblastic leukemia patients in a clinical study [62].

Direct repression of p21 by binding to the proximal promoter has been revealed for Tbx2, a T-box transcription factor, which has already been described as a suppressor of senescence via repression of p19ARF. Tbx2 binds to a T-element (AGGTGTGA) at position -10 on the p21 promoter and thereby represses p21 [72].

Evidence for a direct repression of p21 in Ewing sarcoma cell lines by binding of EWS-FLI1 to a putative ETS transcription factor binding site within the p21 promoter, was published [73], but is contrary to our finding, which strongly indicates the involvement of p53 in the regulation of p21 expression by EWS-FLI1 [74].

1.7 Notch pathway

The notch pathway plays an essential role in many biological processes, like proliferation, differentiation, apoptosis, pattern formation or stem cell maintenance. It has the ability to function as a tumor suppressor as well as an oncogene [75-78].

The four Notch receptors (Notch1-4) and five ligands (Jagged1-2 and Delta like 1,3,4) in mammals are single-pass transmembrane proteins with large extracellular domains mainly consisting of epidermal growth factor like repeats [75]. After interaction of receptor and ligand between neighboring cells, the ADAM (A disintegrin

and A metalloprotease) family protease TACE (TNF-α-converting enzyme) cleaves the receptor, followed by a second cleavage by γ-Secretase and the release of the notch intracellular domain (NICD, ICD, NotchIC). NICD translocates to the nucleus and binds the transcription factor CBF1 (CSL, RBP-Jκ) in that way displacing co-repressors and replaces them with transcriptional co-activators, like Mastermind or the histone acetyltransferase p300 and initiates the transcription of target genes such as Hes and Hey family of genes [76, 78]. Hes1, like Hes5 is involved in the notch dependent inhibition of neuronal development, but Hes1 is as well implicated in pancreatic development. Hey2 is involved in cardiac development and like Hey1 in vascular development [78].

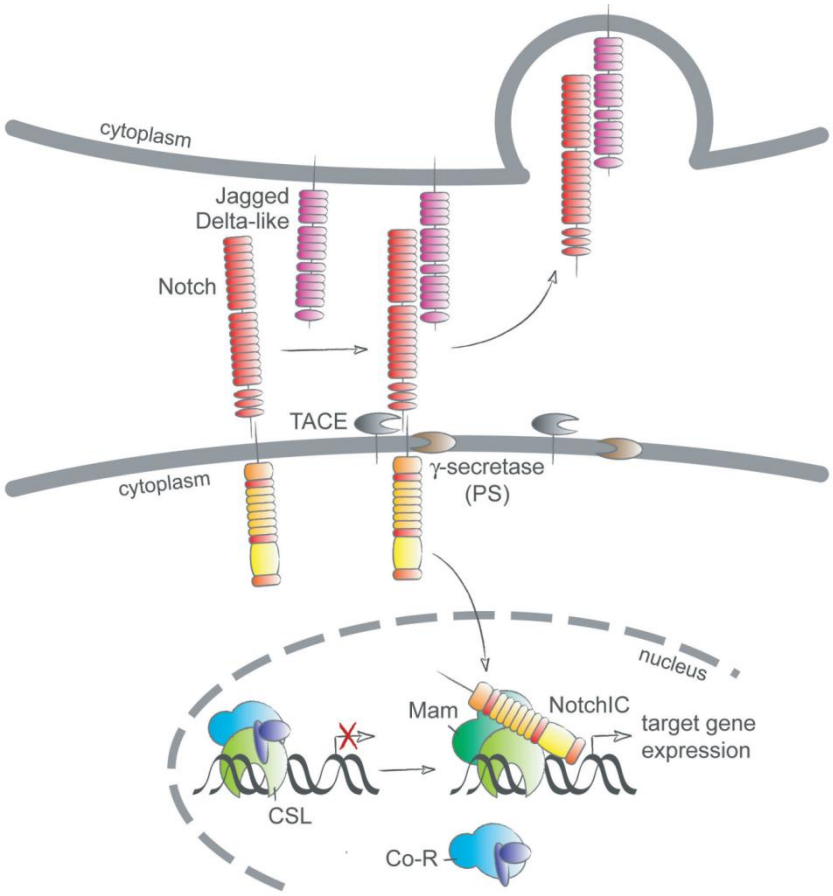

Fig. 7: The Notch pathway; TACE: TNF-α-converting enzyme, PS: presenilin, Mam: Mastermind, NotchIC: Notch intracellular subunit, Co-R: co-repressors [75]

As mentioned above Notch can either act as an oncogene or as a tumor suppressor. Its oncogenic function in T-ALL was discovered when the t(7;9)(q34;q34.3) translocation was revealed to be a fusion of Notch1 with the T-cell receptor beta, leading to the expression of intracellular Notch1 under the regulation of TCR-β and

thereby independent of ligand stimulation. Notch is in general down regulated in the double positive ($CD4^+CD8^+$) T-cell stage Mouse experiments showed that enforced expression of intracellular Notch1 leads to accumulation of cells at this stage and therefore blocking differentiation. The increase in the number of T-cells in the double positive stage favors the accumulation of additional mutations. Furthermore it has been revealed that t(7;9) negative T-ALLs contain point mutations in the Notch1 gene in more than the half of the tumors [76]. Notch4 was discovered to play a role in breast cancer development in mice [79]. In 50% of human mammary carcinomas expression of Numb, a negative regulator of Notch signaling was lost indicating the oncogenic effect of Notch in human breast cancers [80]. Overexpression of several components of the Notch pathway were found in various tumors, like medullablastoma, prostate cancer, renal cell carcinoma, multiple myeloma, Hodgkin's and anaplastic lymphoma [76]. So far the role of Notch remains unclear, for example it is supposed that Notch might act as a tumor suppressor in prostate cancer, since activated Notch is able to slow down the growth of prostate cancer [79]. The tumor suppressive function of Notch1 might be mediated by several mechanisms to induce cell cycle arrest and differentiation. In murine keratinocyctes Notch1 signaling induces the expression of early differentiation markers, like keratin1 or involucrin and activates NF-κB and the onset of terminal differentiation. Notch1 is also able to induce p21 in basal cells and thereby cause cell cycle arrest. Mice lacking Notch1 develop basal-cell-carcinoma like tumors and human basal-cell-carcinoma have decreased levels of Notch1, Notch2 and Jagged1 [81]. We have recently demonstrated that Notch might act as a tumor suppressor in Ewing's sarcoma. Knockdown of EWS-FLI1 resulted in upregulation of Jagged1, thereby activation of the Notch pathway and an increase of Hey1, which down modulated MDMX and led to p53 accumulation [74].

1.7.1 Jagged1

Jagged1 is one of five ligands for the Notch receptor. Following interaction of Jagged1 with a Notch receptor, the Notch pathway is initiated [75]. Mutations of JAG1 are associated with the Alagille syndrome, an autosomal dominant disorder that affects structures in the heart, eye, liver, skeleton, kidney, face and other organs [82]. Jagged1 is furthermore essential for vascular remodeling and endothelium specific JAG1 deletion causes embryonic lethality and cardiovascular disorders [83].

Affymetrix array data and RT-PCR of several Ewing's sarcoma cell lines showed a significant increase of JAG1 upon knock down of EWS-FLI1. As described before, this may lead to the Notch dependent suppression of p53 and its target genes and therefore favors tumor genesis [74].

1.7.2 Hey1

Hey1 belongs to the superfamily of helix-loop-helix (bHLH)-type transcription factors and serves as a transcriptional repressor [84]. Hey1 is a target gene of the Notch pathway determining cell fate decisions. Hey1 is involved in proliferation, migration, blood vessel formation and network formation of endothelial cells. During early stages of angiogenesis Hey1 is down regulated thereby allowing cells to migrate and proliferate, but is enhanced in a later stage to repress VEGFR2 leading to tube formation and establishment of mature vessel phenotype [85]. It was revealed that Hey1 is able to transcriptionally repress MDM2 in colon carcinoma cells [84] and to down regulate MDMX in Ewing's sarcoma cells [74] and thereby lead to an accumulation of p53.

1.8 Aim of the thesis

Using Affymetrix gene chip technology, our lab had analyzed the expression of 14000 genes and searched for genes that are consistently overexpressed (by at least a factor of 2) in the presence of Ews-Fli1. We found 73 such genes but remarkably, we found that Ews-Fli1 also represses by two folds or more transcription of 52 genes in all six Ewing's sarcoma-derived cell lines tested. Whereas it is not known how many of the activated and of the repressed genes are direct transcriptional targets of Ews-Fli1, these data indicate that Ews-Fli1 may act not only as transcriptional activator, but also as a transcriptional repressor.

Among these we found the cell cycle inhibitor p21, the Notch ligand Jagged1 and the Notch target Hey1 to be repressed by EWS-FLI1. The mechanisms of the transcriptional repression of p21, Jagged1 and Hey1, should be studied within this thesis. To study p21, 10kb of the promoter region should be amplified in 14 overlapping fragments and cloned into pGL4.10 or pGL4.10-pTK. The activity of those gene reporter constructs should be studied in a p53wt and a p53mut Ewing’s sarcoma cell line in the presence and absence of EWS-FLI1 and upon induction of p53 with etoposide or modulation of p53 with shRNA.

Similarly JAG1 and Hey1 promoter reporter constructs should be established and tested for EWS-FLI1 dependent activity.

2 Material and Methods

2.1 Material

2.1.1 Media

Luria Broth (LB):

1% Trypton, 1% NaCl, 0,5% Yeast-extract. LB has to be sterilized in the autoclave.

For Agar Dishes: 1% Trypton, 1% NaCl, 0,5% Yeast extract, 1,5% Agar

Ampicillin was supplied after cooling down the autoclaved agar to 50°C.

Pour out the solution into petri-dishes.

Terrific Broth (TB):

To 900ml H_20 add 12g Tryptone, 24g Yeast-extract and 4ml Glycerol. Autoclave as well 100ml of 0,17M KH_2PO_4, 0,72M K_2HPO_4, afterwards mix both solutions.

NZY+ Broth:

10 g of NZ amine (casein hydrolysate)

5 g of yeast extract

5 g of NaCl

pH 7.5

per liter

sterilization by autoclaving

the following filer-sterilized supplements were added prior to use:

12.5 ml of 1 M MgCl2

12.5 ml of 1 M MgSO4

20 ml of 20 % (w/v) glucose

RPMI 1640 with GlutaMAX^{Tm}-I: Invitrogen, Groningen, Netherlands

Add 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

Opti-MEM: Invitrogen, Groningen, Netherlands

DMEM: Invitrogen, Groningen, Netherlands

1000 mg/L glucose, 4mM L-glutamine and 110 mg/L sodium pyruvate

Add 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100000 Units/l penicillin / streptomycin (PAA Laboratories, Linz, Austria)

Trypsin / EDTA: PAA Laboratories, Linz, Austria

Accutase: PAA Laboratories, Linz, Austria

Puromycin: Sigma, St. Louis, USA

Etoposide: Sigma, St. Louis, USA

γ- secretase inhibitor: Calbiochem, Merck, Darmstadt, Germany

Ampicillin: Biomol, Hamburg, Germany

Doxycycline: Sigma, St. Louis, USA

Blasticidin: Invitrogen, Groningen, Netherlands

Zeocin: Cayla, Toulouse, France

2.1.2 Buffers

PBS: 137mM NaCl; 3mM KCl; 6,5mM Na_2HPO_4-$2H_2O$; 1,5mM KH_2PO_4

TBS: 50mM Tris, 150mM NaCl, pH 7,5

TBS-T: 50mM Tris, 150mM NaCl, 0,1% Tween 20; pH 7,5

2x sample buffer:

20% (v/v) glycerol

6% ß-mercaptoethanol

3% SDS

125mM Tris-Cl pH 6,8

a few bromphenol blue crystals

Laemmli buffer:

15,1g Tris

72g glycine

25ml 20% SDS

per 1 liter

Transfer buffer:

14g glycine

3g Tris

20% methanol

per 1 liter

Ponceau S staining solution (10x stock):

2g Ponceau S

30g trichloroacetic acid

30g 5-sulfosalicylic acid

ad 100ml

Loading Dye:

4M Urea

80mM EDTA

10% Saccharose

0,25% BPB

TBE:

5,4g Tris Base

2,75g Boric Acid

2ml 0,5M EDTA/pH8

per 1 liter

Blocking solution:

10% blocking reagent (Roche, Basel, Switzerland) in maleic acid buffer (100mM Maleic Acid, 150 mM NaCl, pH= 7.5, sterile).
Heat to dissolve and autoclave.

2.1.3 Bacterial strains

JM109: *end*A1, *rec*A1, *gyr*A96, *thi*, *hsd*R17 (r_k^-, m_k^+), *rel*A1, *sup*E44, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB, *lacI*^{q}ZΔM15], (Promega, Madison, USA)

Sure2 Supercompetent Cells: e14-(McrA-) Δ*(mcrCB-hsdSMR-mrr)171 endA1 gyrA96 thi-1 supE44 relA1 lac recB recJ sbcC umuC*::Tn*5* (Kanr) *uvrC* [F' *proAB lacI*q*Z*Δ*M15* Tn*10* (Tetr) Amy Camr], (Stratagene, La Jolla, USA)

2.1.4 Cell lines

SK-N-MC: Ewing tumor cell line established by J. Biedler (Memorial Sloan-Kettering Cancer Center, New York, USA) from a pPNET in the rib of 14 year old female child (expresses EWS-FLI1 type 1, truncated p53) (ATCC: HTB-10)

TC252: Ewing tumor cell line established by T. Triche (Department of Pathology, Children's Hospital, Los Angeles, USA) (expresses EWS-FLI1 type 1, p53 wild type) presumably from a female patient.

STA-ET1: Ewing tumor cell line established at the Children's Cancer Research Institute, Vienna (expresses EWS-FLI1 type 1, p53 wild type)

WE68: Ewing tumor cell line established by F. Van Valen (Dept. of Pediatrics, University of Muenster, Germany) (expresses EWS-FLI1 type 1, p53 wild type)

ASP14: Ewing tumor cell line established from A673 parental cell line by Javier Alonso (Laboratorio de Patología Molecular de Tumores Sólidos Infantiles, Departamento de Biología Molecular y Celular del Cáncer, Instituto de Investigaciones Biomédicas, Madrid, Spain) (expresses Ews-Fli1 type 1, p53 mutant). Inducible cell line, Doxycycline induces shRNA against Ews-Fli1

HepG2: Hepatocarcinoma cell line established from a 15 year old Caucasian boy. (p53 wildtype)

2.1.5 Plasmids

2.1.5.1 Existing plasmids

pGL4.10 - TK-RL: TK driven mammalian expression vector encoding Renilla Luciferase, used as a transfection efficiency control. (constructed by Idriss Bennani-Baiti, CCRI, Vienna)

pCMV: CMV promoter based mammalian expression (constructed by Suzanne Baker, John's Hopkins, Baltimore)

pCMV Ews-Fli type II: CMV promoter based mammalian expression vector encoding Ews-Fli type II. (Gift from Mark Ladani, Departments of Pathology, Medicine, and Surgery, and the Human Oncology and Pathogenesis Program, Memorial Sloan-Kettering Cancer Center, New York, USA)

pSuperΔRV: pSUPER-based retroviral mammalian expression vector. (Gift from Reuven Agami, Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands)

pSuperΔRVsh22: mammalian expression vector encoding shRNA against Ews-Fli type II. (constructed by Jozef Ban, CCRI, Vienna)

pSuperΔRVsh30: mammalian expression vector encoding shRNA against Ews-Fli type I. (constructed by Jozef Ban, CCRI, Vienna)

pSuper-shp53: mammalian expression vector encoding shRNA against p53. (Gift from Reuven Agami, Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands)

Pst-Neg: mammalian expression vector encoding scrambled shRNA (Gift from Dr. B. Kaminska, Laboratory of Transcription Regulation, Department of Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland)

RPCIB753F15397Q2: p21 expressing pBACe3.6 Cosmid (RZPD German Resource Center for Genome Research, Berlin, Germany).

RZPDB737F0934D6: Jag1 expressing pBACe3.6 Cosmid (RZPD German Resource Center for Genome Research, Berlin, Germany).

Hey1-FL: mammalian expression vector encoding 3,9kb of the Hey1 Promoter. (Gift from Erwin Böttinger, Department of Molecular Genetics and Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA)

Hey1-M5: mammalian expression vector encoding 1.7kb of the Hey1 Promoter. (Gift from Erwin Böttinger, Department of Molecular Genetics and Department of Medicine, Albert Einstein College of Medicine, Bronx, New York, USA)

pGL4.10-Jag1-459-1713: mammalian expression vector encoding Jagged1 Promoter sequence bp 459 to 1713 (constructed by Cornelia Schuh, CCRI, Vienna)

2.1.5.2 Cloning Plasmids

pGL4.10: Promega, Madison, USA

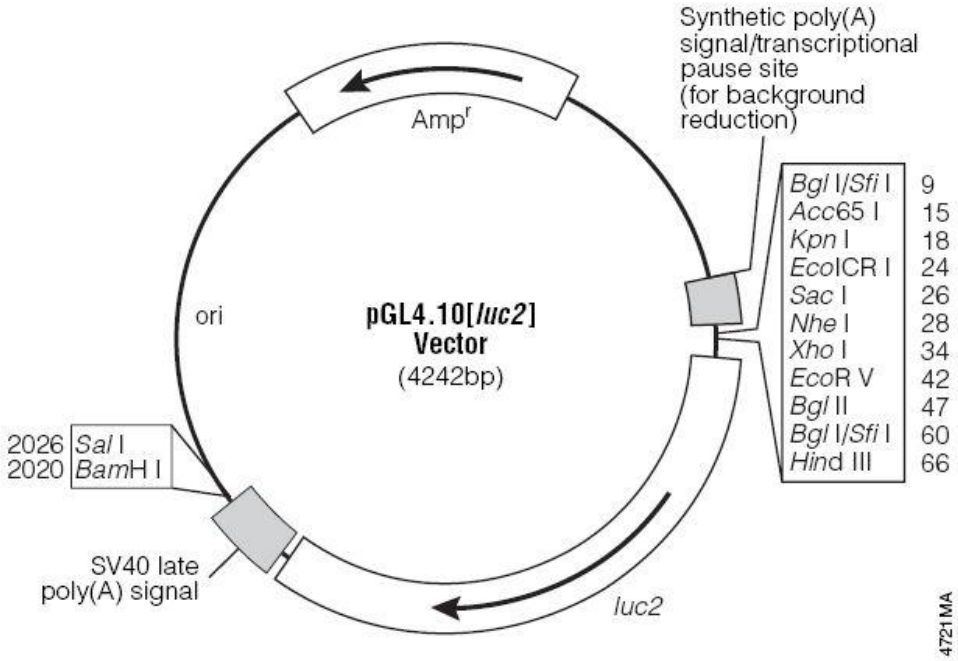

Fig. 8: pGL4.10

pGL4.10 - TK: TK driven mammalian expression vector encoding Firefly Luciferase.

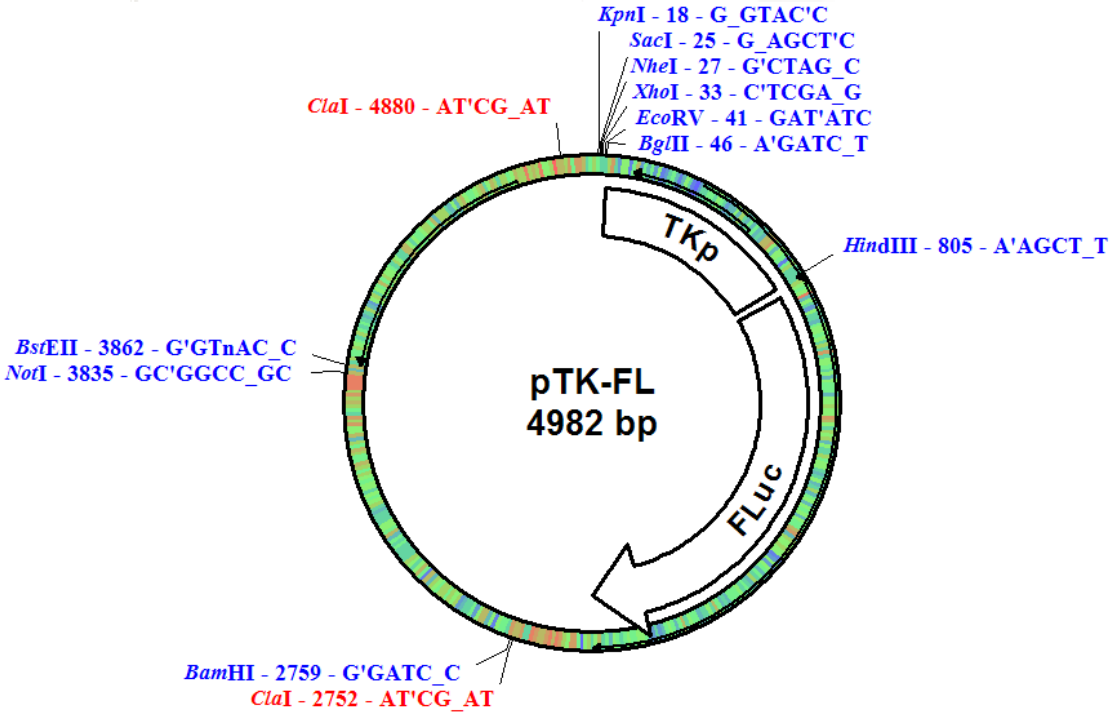

Fig. 9: pGL4.10-pTK

2.1.5.3 Plasmids established in the course of this thesis

pGL4.10 - 146/147: p21 reporter gene construct that encodes the p21 Promoter Region 1- 599bp

pGL4.10 - TK- 147/148: TK driven p21 reporter gene construct that encodes p21 Promoter Region 1-1144bp

pGL4.10 - TK- 149/150: TK driven p21 reporter gene construct that encodes p21 Promoter Region 494- 1048bp

pGL4.10 - TK- 151/152: TK driven p21 reporter gene construct that encodes p21 Promoter Region 848- 2135bp

pGL4.10 - TK- 153/154: TK driven p21 reporter gene construct that encodes p21 Promoter Region 2019- 2656bp

pGL4.10 - TK- 175/176: TK driven p21 reporter gene construct that encodes p21 Promoter Region 2538- 3717bp

pGL4.10 - TK- 172/173: TK driven p21 reporter gene construct that encodes p21 Promoter Region 2591- 4186bp

pGL4.10 - TK- 170/171: TK driven p21 reporter gene construct that encodes p21 Promoter Region 3983- 5050bp

pGL4.10 - TK- 168/169: TK driven p21 reporter gene construct that encodes p21 Promoter Region 4899- 5724bp

pGL4.10 - TK- 166/167: TK driven p21 reporter gene construct that encodes p21 Promoter Region 5470- 6699bp

pGL4.10 - TK- 164/165: TK driven p21 reporter gene construct that encodes p21 Promoter Region 6491- 7457bp

pGL4.10 - TK- 162/163: TK driven p21 reporter gene construct that encodes p21 Promoter Region 7296- 7605bp

pGL4.10 - TK- 160/161: TK driven p21 reporter gene construct that encodes p21 Promoter Region 7044- 9043bp

pGL4.10 - TK- 158/159: TK driven p21 reporter gene construct that encodes p21 Promoter Region 8930- 10004bp

pGL4.10 –Jag1-573: Jag1 reporter gene construct that encodes 573bp of the Jag1 Promoter region.

pGL4.10 –Jag1-1713: Jag1 reporter gene construct that encodes 1.7kb of the Jag1 Promoter region.

2.1.6 Oligonucleotides

p21- Constructs:

p21-1- 599 and 1- 1144bp forward primer:	*gggtacc*CTGTGAAATAAACGGGACTGA
p21- 1- 599 reverse primer:	*ggctagcc*AGCGCGGCCCTGATATAC
p21- 1- 1144 reverse primer:	*gggtaccc*TGGGAGCGGATAGACACATC
p21- 494- 1048 forward primer:	*gggtaccc*ATCTGCAAATGAGGGTTA
p21- 494- 1048 reverse primer:	*ggctagc*CTGATCCCTCACTAGGTCA
p21- 848- 2135 forward primer:	*gggta*CCCAGGTAAACCTTAGCCTCTT
p21- 848- 2135 reverse primer:	*ggctagcc*TACTCCCCACATAGCCCGTA
p21- 2019- 2656 forward primer:	*gggtacc*CTGCCTCTGCTCAATAATGTT
p21- 2019- 2656 reverse primer:	*ggctagc*CTGACTCCCAGCACACACTC
p21- 2538- 3717 forward primer:	*gggtaccc* AGGGGACCGTGTCTGGAGGA
p21- 2538- 3717 reverse primer:	*ggctagcc* GGGGAGTCCCAAATAGGGGCAGT
p21- 2591- 4186 forward primer:	*gggtaccc* TAGGCAGCCCCAATGCAGACA
p21- 2591- 4186 reverse primer:	*ggctagcc* GCTCCCAAGAAGTGAGACCCC
p21- 3983- 5050 forward primer:	*gggtaccc* TGGGAGACCGAGGCAGAC
p21- 3983- 5050 reverse primer:	*ggctagcc* TCACAGAGGGGCCAGACCTA
p21- 4899- 5724 forward primer:	*gggtaccc* GCCAGGCGTGGTGGTTCGT
p21- 4899- 5724 reverse primer:	*ggctagcc* AGACCCCGGCTCCCGAAAC

p21- 5470- 6699 forward primer:	*gggtaccc* TCAGGTGGCCTGTGTTCA
p21- 5470- 6699 reverse primer:	*ggctagcc* GGAATGCAATGGTGC
p21- 6491- 7457 forward primer:	*gggtaccc* GGCTCGAGAATTTCACCTGGCCT
p21- 6491- 7457 reverse primer:	*ggctagcc* CCTCGGGCAAGAGAGCTTCC
p21-7296- 7605 forward primer:	*gggtaccc* TATCCCATAGAAGTTGGTGGGGC
p21- 7296- 7605 reverse primer:	*ggctagcc* AAGACAGTAATGCTCAGCCA
p21- 7044- 9043 forward primer:	*gggtaccc*GACCTGGAGGGGGTGTCT
p21- 7044- 9043 reverse primer:	*ggctagcc*AGGGTCATTTTTGGCCTGTA
p21- 8930- 10004 forward primer:	*gggtaccc*GAACGTGGGTGGGAGATG
p21- 8930- 10004 reverse primer:	*ggctagcc*TCAACGATTGTGACACCTG

Jag1- constructs:

JAG1-F:	*tcga*AGATCTAGGGGCGTGCCCAGGGTGAG
JAG1-R:	*tcga*AAGCTTCGCTGCGCCGCGCGCCGC

Site directed mutagenesis primer:

151/152-Ets1-mut	GGTCAGCTGCGTTAGAAAAAGAAGACTGGGCATGTCTGGG
151/152-Ets1-mut	CCCAGACATGCCCAGTCTTCTTTTTCTAACGCAGCTGACC
151/152-p53-mut	GAGGAAGAAGACTGGGCACCTCTGGGCAGAG
151/152-p53-mut	CTCTGCCCAGAGGTGCCCAGTCTTCTTCCTC
153/154-p53-mut	GGCCGTCAGGAACACCTCCCAAGGTGTTGAGCTCTGGC
153/154-p53-mut	GCCAGAGCTCAACACCTTGGGAGGTGTTCCTGACGGCC
153/154-p53-mut	GCCTGCTTCCCAGGAACACCCTTGGGCAGCAGGC
153/154-p53-mut	GCCTGCTGCCCAAGGGTGTTCCTGGGAAGCAGGC

153/154-Ets1mut	GTTCCCAGCACTCTCTCTCCCTTCCTAGGCAGC
153/154-Ets1mut	GCTGCCTAGGAAGGGAGAGAGAGTGCTGGGAAC

MWG pGL-series Standard primers:

pGL rev:	CTTTATGTTTTTGGCGTCTTCC
pGL3 for:	CTAGCAAAATAGGCTGTCCC

Self-designed pGL4-pTK sequencing primer:

pTK rev:	CTAACCACCGCTTAAGCG
pTK-147/148 internal:	ACTTCGTGGGGAAATGTGTC
pTK-160/161 internal:	AGCCAGGCTTCGTGGTGTG
pTK-172/173 internal:	GCACAATCTCAGCTCACTGC

2.1.7 Antibodies

Anti-p21 (F-5): Mouse monoclonal antibody against amino acids 1-159 representing full length p21. (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-6246).

Dilution: 1:70

Anti-p53 (DO-1): Mouse monoclonal antibody against the C-terminus of p53 (B. Vojtesek, Masaryk Memorial Cancer Institute, Brno, Czech Rep.)

Dilution: 1:5

Anti-Fli-1 (C-19): Rabbit polyclonal antibody against the C-terminus of FLI1 (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-356).

Dilution: 1:100

Anti-Jagged1 (H-114): Rabbit polyclonal antibody against amino acids 1110-1223 of Jagged 1 (Santa Cruz Biotechnology Inc., Santa Cruz, USA, sc-8303).

Dilution: 1:200

Anti-Rabbit IgD, DyLight^{TM}800: Goat Anti-Rabbit antibody reacts with heavy chains of rabbit IgG and with light chains of most rabbit immunoglobulins (Pierce Biotechnology Inc., Rockford, USA).

Dilution: 1:20000

Anti-Mouse IgD, DyLight^{TM}800: Goat Anti-Rabbit antibody reacts with heavy chains of mouse IgG and with light chains of most mouse immunoglobulins (Pierce Biotechnology Inc., Rockford, USA).

Dilution: 1:20000

2.2 Methods

2.2.1 DNA Methods

2.2.1.1 Cloning of p21 constructs

PCR was performed for 20cycles in a total volume of 50µl with 20pmol of the corresponding primers, 10mM dNTPs (Promega, Madison, USA), 5µl 10xPfu Buffer (Promega, Madison, USA), 1µl Pfu (Promega, Madison, USA) and 100ng of the Cosmid RPCIB753F15397Q2 were used as a template. PCR products were digested with KpnI and NheI (NEB, Ipswich, USA) using Buffer1 in the presence of BSA, over night at 37°C. Gel purification was performed with ZymocleanTM Gel Recovery Kit (Zymo Research, Orange, USA). pGL4.10 and pTK were digested with KpnI and NheI and dephosphorylated with CIAP (NEB, Ipswich, USA) at 37°C for 30min.

Ligation was performed overnight at RT with T4 Ligase and the provided T4 Ligase Buffer (Promega, Madison, USA).

2.2.1.2 Cloning of Jag1-573 construct

PCR was performed for 20cycles in a total volume of 50µl with 20pmol of the corresponding primers, 10mM dNTPs (Promega, Madison, USA), 5µl 10xPfu Buffer (Promega, Madison, USA), 1µl Pfu (Promega, Madison, USA) and 100ng of the Cosmid RZPDB737F0934D6 were used as a template. The 573bp PCR product was digested with BglII and HindIII(NEB, Ipswich, USA) using Buffer 2 over night at 37°C. Gel purification was performed with ZymocleanTM Gel Recovery Kit (Zymo Research, Orange, USA). pGL4.10 was digested with BglII and HindIII and dephosphorylated with CIAP (NEB, Ipswich, USA) at 37°C for 30min. Ligation was performed overnight at RT with T4 Ligase and the provided T4 Ligase Buffer (Promega, Madison, USA).

2.2.1.3 Cloning of Jag1-1713 construct

pGL4.10-Jag1-573 and pGL4.10-Jag1-459-1713 were digested with BglII and AseI (NEB, Ipswich, USA) using Buffer 3 at 37°C over night. Digested pGL4.10-Jag1-573 was dephosphorylated with CIAP (NEB, Ipswich, USA) at 37°C for 30min and, as well as the digested pGL4.10-Jag1-459-1713 run on an 1% Agarose Gel. Gel purification was performed with ZymocleanTM Gel Recovery Kit (Zymo Research, Orange, USA). Ligation was performed overnight at RT with T4 Ligase and the provided T4 Ligase Buffer (Promega, Madison, USA).

2.2.1.4 Site directed Mutagenesis

Site directed Mutagenesis was performed using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA, 200523) according to manufacturer's instructions.

2.2.1.5 Deletion of Cbf1 transcription factor binding site in pTK-151/152

pTK-151/152 was digested over night with KpnI and NsiI (NEB, Ipswich, USA) using Buffer 2 and BSA at 37°C. After gel purification using ZymocleanTM Gel Recovery Kit (Zymo Research, Orange, USA) ligation was performed overnight at RT with T4 Ligase and the provided T4 Ligase Buffer (Promega, Madison, USA).

2.2.1.6 Transformation of competent E.coli (JM109)

Mix DNA and 40µl of competent cells.

Keep the mixture 30min on ice

Heat shock the cells 60sec at 42°C.

Keep the mixture 2min on ice.

Add 400µl LB and keep the cells on 37°C for 30min.

Plate the suspension on antibiotic-plates and incubate over night at 37°C.

2.2.1.7 Transformation of competent E.coli (Sure2 Supercompetent Cells)

Thaw 100µl of competent cells on ice.

Add 2µl β-ME.

Keep the mixture 10min on ice, swirl every 2min.

Add DNA.

Incubate on ice 30min.

Heat shock the cells 30sec at 42°C.

Keep the mixture 2min on ice.

Add 900µl of preheated NZY Broth and keep the cells on 37°C for 1h.

Plate the suspension on antibiotic-plates and incubate over night at 37°C.

2.2.1.8 Mini Prep

2ml of 10mg/ml ampicillin containing LB were inoculated with a single colony and incubated over night at 37°C shaking. For Mini Preps the Quiagen MiniPrep kit (Quiagen, Austin, USA) was used according to the manufacturer's instructions.

2.2.1.9 Restriction digest p21-plasmids

500ng DNA of p21-plasmids was digested with KpnI and NheI in Buffer1 (NEB, Ipswich, USA) and the presence of BSA at 37°C for 4h. Restriction reactions were analyzed on an 1%Agarose Gel. Plasmids containing the corresponding fragment were sent for sequencing.

2.2.1.10 Restriction digest jagged1 plasmids

Jagged1 plasmids were digested with BglII and HindIII in Buffer 2 (NEB, Ipswich, USA) at 37°C for 4h and run on an 1%Agarose Gel. Plasmids containing the corresponding fragment were sent for sequencing.

2.2.1.11 Sequencing

Sequencing was done at MWG Biotech (Ebersberg, Germany) or VBC-Biotech Research GmbH (Vienna, Austria). The sequences were first blasted against the NCBI human genomic plus transcript database. In a further attempt the actual sequences were aligned in a multiple sequence alignment, with the NCBI reference sequence (>ref|NT_007592.14|Hs6_7749 07.01.08), the celera sequence (>ref|NW_923073.1|HsCraAADB02_255 Homo sapiens chromosome 6 genomic contig, alternate assembly based on Celera assembly), an older version oft he NCBI reference sequence (02.07) and the NCBI chimp p21 sequence (>ref|NW_001236525.1|Ptr6_WGA7082_2:1554585-1569585 Pan troglodytes chromosome 6 genomic contig, reference assembly (based on Pan_troglodytes-2.1) 07.01.08) using ClustalW (www.ebi.ac.uk/tools/clustalw2/index.html).

All alterations that could be found were analyzed with Genomatix and TFBS (http://www.cbrc.jp/research/db/TFSEARCH.html) see whether Transcription factor binding sites were lost or gained. (See also in “Appendix”) Transcription factor binding sites were defined to be significant, if they had a matrix similarity score 0.95 or higher in Genomatix.

2.2.1.12 Maxi Prep

250ml of 10mg/ml ampicillin containing LB were inoculated with a preculture of the corresponding plasmid and incubated over night at 37°C shaking. For Maxi Preps the Quiagen Endotoxin free MaxiPrep kit (Quiagen, Austin, USA) was used according to the manufacturer’s instructions.

2.2.2 Protein Methods

2.2.2.1 SDS- Polyacrylamid Gel Electrophoresis

The SDS- polyacrylamid gel consists of two different layers of gels, the stacking gel which is always a 6% gel and the separating gel which varies between 6 and 12,5%.

Separating gel:

	6%	**8,5%**	**12,5%**
30%Acrylamid / 0,8% Bis	1,05ml	1,4ml	2,1ml
H_2O	2,625ml	2,275ml	1,575ml
1,5M Tris pH8,8	1,25ml	1,25ml	1,25ml
20% SDS	25µl	25µl	25µl
10% APS	50µl	50µl	50µl
TEMED	6µl	6µl	6µl

Stacking gel:

30%Acrylamid / 0,8% Bis	415µl
H_2O	1,7ml
1M Tris pH6,8	315µl
20% SDS	12,5µl
10% APS	25µl
TEMED	2,5µl

The cell pellet was resuspended in PBS and the same amount of 2x Sample Buffer was added. After boiling the samples 10min at 95°C and centrifugation they were loaded on the gel, which was run at 40mA till the bromophenol blue front was not visible any more.

2.2.2.2 Western Blot

The transfer unit consisting of a sponge, three 3 pieces of 3MM paper, the gel, a nitrocellulose membrane, three 3 pieces of 3MM paper and a sponge (everything presoaked with transfer buffer), was put in the blotting stock. The transfer was run at 400mA for 90min on ice.

For a first staining the membrane was incubated in PonceauS solution for 5 to 10min and scanned afterwards. To block unspecific binding, the membrane was incubated in 1% blocking solution for 1 hour RT. The primary antibody was diluted in 0,5% blocking solution, added to the membrane and incubated over night at 4%. The membrane was then washed twice with TBST for 10min and once with 0,5% blocking solution. The secondary antibody was diluted in 0,5% blocking solution, added to the membrane and incubated for 1 hour RT: After washing the membrane three times with TBST and once with PBS for 15min each, it was scanned using the Li-cor Odysee Infrared Imaging System (Li-cor Biosciences, Lincoln, USA).

2.2.3 Cell culture techniques

Human cell lines were routinely grown in RPMI 1640 GlutaMAX^{Tm}-I medium (Invitrogen, Groningen, The Netherlands) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories, Linz, Austria) and 100.000 U/l penicillin/streptomycin (PAA Laboratories, Linz, Austria) in 5% CO_2 at 37°C.

2.2.3.1 Transfection

Cells were seeded in a 24well plate to 50-80% confluency one day before transfection. RPMI medium was removed and cells were incubated in Opti-MEM I at least one hour prior to transfection. Transfection was performed with Lipofectamine and Plus reagent (Invitrogen, Groningen, The Netherlands) according to the manufacturer's instructions in serum-free OptiMEM I medium (Invitrogen, Groningen, The Netherlands) using 50ng of the reporter gene plasmid, 50ng of pTK-RL and 150ng of shRNA per well. After incubation for 4 hours the serum-free medium was replaced by supplemented RPMI medium.

2.2.3.2 Reporter Gene Assays

For p21 and Hey1 experiments gene repoter assays were performed 72h after transfection for Jagged1 96h after transfection. Reporter Gene Assays were performed using the Dual-Glo^{TM} Luciferase Assay System (Promega, Madison, USA, E2920) according to the manufacturer's instructions.

2.2.3.3 Etoposide Treatment

Etoposide (Sigma, St. Louis, USA) was used in a final concentration of 1µM and cells were incubated for 16h.

3 Results:

3.1 Analysis of transcriptional repression of p21 – strategy

To analyze the repression of Ews-Fli1 on p21, 14 fragments of the p21 promoter and 10kb upstream were cloned into a luciferase reporter vector. These p21-fragment containing plasmids were then transfected into TC252, a p53 wildtype and SKNMC a p53 mutant Ewing's Sarcoma cell line, in the presence and absence of Ews-Fli1 and tested in reporter gene assays. In further experiments, using the same setting as mentioned before, either p53 was induced via Etoposide treatment or knocked down via shp53.

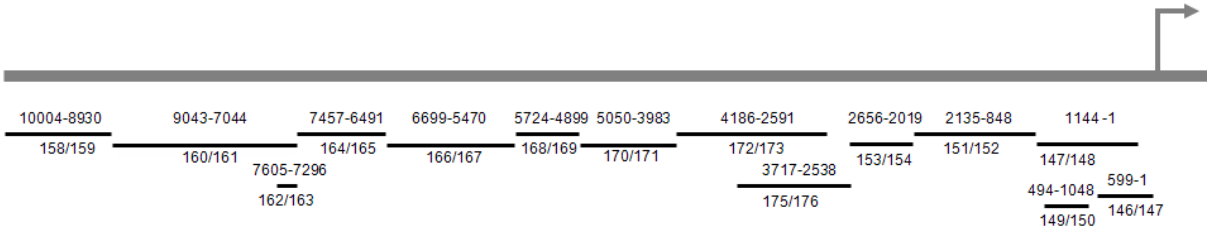

Fig. 10: P21 fragments, ↱: transcription start site

3.1.1 Cloning of p21 fragments

As a cloning vector we chose the luciferase reporter vector pGL4.10 from Promega (see also "Material and Methods"). Since pGL4.10 is lacking a promoter, only the p21-promoter containing fragment was inserted into this vector, whereas the other fragments were cloned into pGL4.10-pTK using herpes simplex virus thymidine kinase as a promoter. TK as a promoter was chosen, because of its moderate levels of expression.

In a first attempt genomic DNA was used as a template for the PCR of the fragments. Since it was difficult to amplify, a high cycle number was required, which resulted in various mutations. After this finding, we tried unsuccessfully to find a plasmid containing 10kb of p21 in the literature, but we found a commercially available cosmid containing at least 10kb of p21 5'flanking sequence, which was then used as a template for PCR. We could then reduce the cycle number to 20 and used as well Pfu proofreading polymerase. Still sequencing revealed various mutations, but since

they were reproducibly found in multiple clones of given constructs, we supposed that this was not an amplification problem, but may constitute polymorphisms.

3.1.2 Sequence analysis

As mentioned before the sequences were blasted against the NCBI human genomic plus transcript database, but as well multiple sequence alignment using the actual insert sequence, the NCBI reference sequence, the celera reference sequence and an other older version of the NCBI reference sequence was performed (see also „Material and Methods“). This analysis revealed that some of the already found misaligned nucleotides were validated as mutations, but that others could be aligned to one or the other reference database sequence, indicating polymorphisms or sequencing misreading. To see whether these misalignments/mutations were evolutionary conserved we performed the same multiple alignment (see also “Appendix”) again, but incorporated also the chimpanzee sequence of p21. After this analysis we finally decided which mutations should be considered to be real polymerism artifacts. Nevertheless all alterations were analyzed with Genomatix and TFBS (see also “Material and Methods”) to see whether Transcription factor binding sites were lost or gained. Since no high score TFBS were changed, we decided to proceed to reporter gene assays with these plasmids.

3.1.3 p21 Reporter Gene Assays

In a first screening all 14 p21-plasmids were transfected into the p53 wildtype TC252 and p53 mutant SKNMC Ewing Sarcoma cell line either in combination with pSuperΔRVsh30 to knock down Ews-Fli1, or with ShScrambled and pSuperΔRV as a control. As a control for transfection efficiency also pTK-RL was co-transfected. The reporter gene assay was done 72h after transfection:

For normalization each of the three different counts for one reporter gene vector containing the corresponding p21 fragment, was divided by the corresponding counts of the pTK-RL plasmid, which was co transfected to measure transfection efficiency. The same procedure was done for pGL4.10 and pGL4.10-pTK to eliminate the background activities associated with these vectors. Additionally the ratio between the empty vector in the presence and absence of EWS-FLI1, was taken care of by dividing it from the normalized counts of the p21 fragments. An average, as well as a standard deviation was then calculated from those numbers. Fold activations were

calculated by dividing the normalized counts in the absence of Ews-Fli1 (sh30) by the normalized counts for the empty vector (ΔRV and shScrambled).

$$Normalized\ Luciferase\ counts = \frac{\frac{FL-P\ (sh30)}{RL\ (sh30)}}{\frac{FL-P\ (\Delta RV)}{RL\ (\Delta RV)}} \Big/ \frac{\frac{FL-E\ (sh30)}{RL\ (sh30)}}{\frac{FL-E\ (\Delta RV)}{RL\ (\Delta RV)}}$$

Fig. 11: Formula for the calculation of normalized luciferase counts. FL-P: Average of firefly luciferase activity coupled to p21 promoter fragment. FL-E: Average of firefly luciferase activity of pGL4 empty vector. RL: Average of Renilla firefly luciferase control vector activity

To get an approximate standard deviation, the following formula was used:: $\%\ std = \frac{\%\ [std\,(x)]}{Avg\ (y)}$.

Fig. 12: Formula for the calculation of standard deviations. X= normalized luciferase counts for knockdown of EWS-FLI1, y= normalized luciferase counts for empty vector.

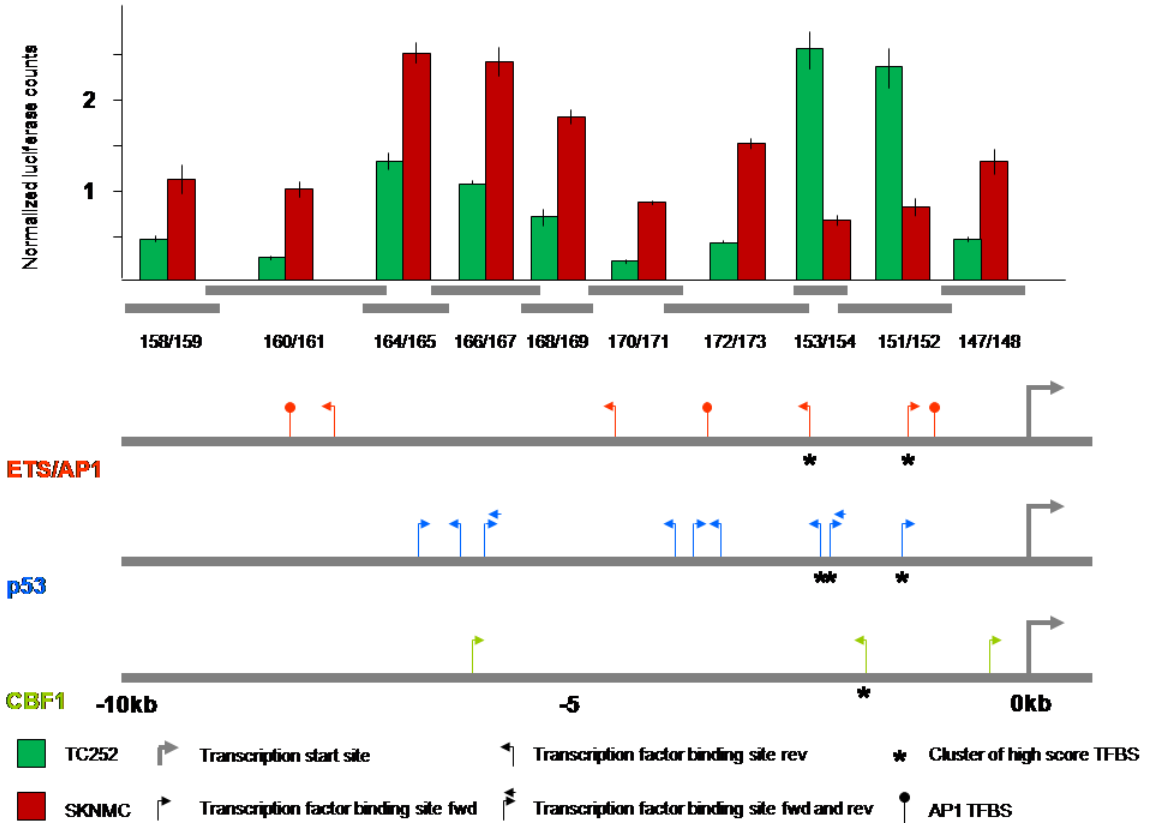

Fig. 13: Basal activity of the various p21 plasmids. Transfections were done in triplicates.

pTK-151/152 and pTK-153/154 showed the highest activity in TC252, while 164/165, 166/167 and 168/169 showed the highest basal activity in SKNMC cells.

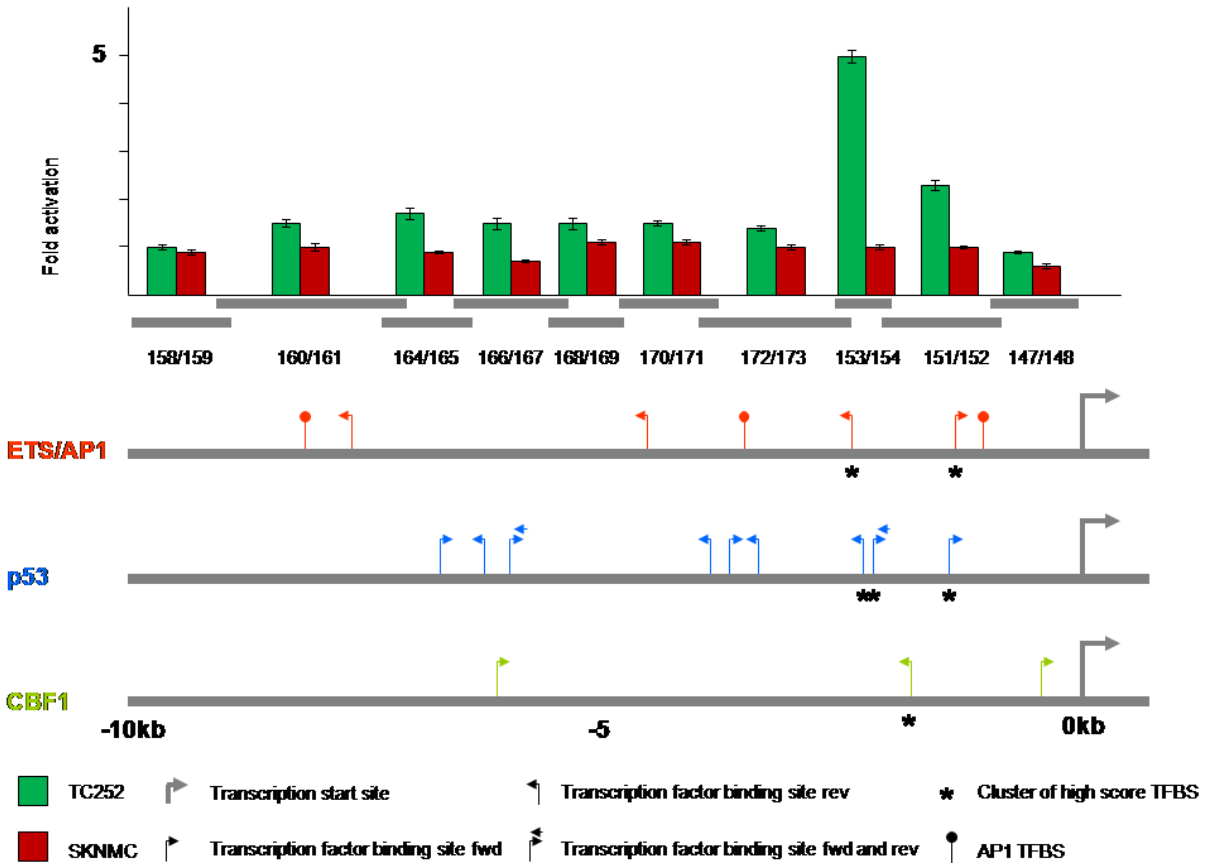

Fig. 14: Fold activation of different p21-fragment-containing plasmids upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30). Transfections were done in triplicates.

Knock down of EWS-FLI1 in TC252 showed a fivefold induction of fragment pTK-153/154 and a 2,3 fold induction of fragment pTK-151/152. Interestingly enough only in these two fragments p53 binding sites together with Ets1 binding sites are present.

In pTK-151/152 the consensus core sequences for p53 (CATG) and Ets1 (GGAA) are separated from each other only by 10bp, indicating a strong possibility of interaction between p53 and EWS-FLI1.

In pTK-153/154 three p53 binding sites within 65bp were found. Although three Ets1 binding sites are present, only the closest one to the p53 cluster, had a high enough score (see also “Material and Methods”) to be taken in account.

These findings together with the fact, that SKNMC cells, which are p53 mutant, do not show an induction upon knock down of EWS-FLI1, suggest that p53 is involved in the repression of p21 via EWS-FLI1.

In parallel, cells transfected with p21-plasmids in the presence and absence of EWS-FLI1 were treated with Etoposide 56h after transfection to induce p53, 16h later reporter gene assays were performed.

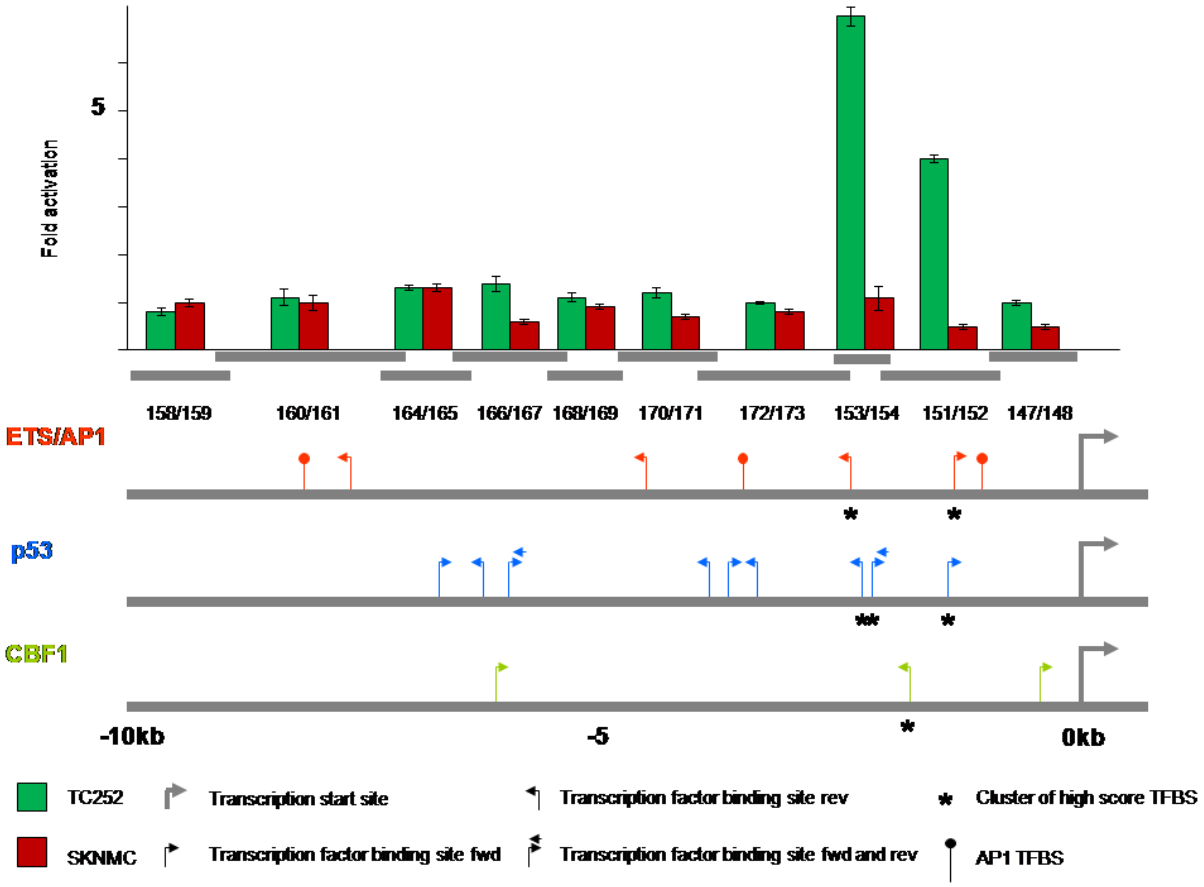

Fig. 15: Fold activation different p21-fragment-containing plasmids upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30). and induction of p53 via Etoposide treatment. Transfections were done in triplicates.

pTK-153/154 and pTK-151/152 have already shown a high induction of p21 after EWS-FLI1 knock down, which was even stronger when at the same time, p53 was induced. pTK-153/154 shows a seven fold induction in TC252, whereas no effect is visible in the p53 mutant cell line SKNMC. pTK-151/152 shows a fourfold increase in TC252 and a slight reduction in SKNMC. Although several other fragments contain p53 binding sites as well, only those two including binding sites for p53 as well as Ets1, show an induction upon knock down of EWS-FLI1 and p53 induction, indicating again a strong interaction between those two transcription factors.

In a further experiment the most promising candidates were tested in a reporter gene assay in the presence and absence of EWS-FLI1 and p53, using shRNA against p53. Beside pTK-151/152 and pTK-153/154, which have already shown a significant effect, pTK-164/165 and pTK-147/148 where at least a small effect was observed, were tested in this assay. For more reliable results, this time the transfections were done in sextuplicates instead of triplicates as before.

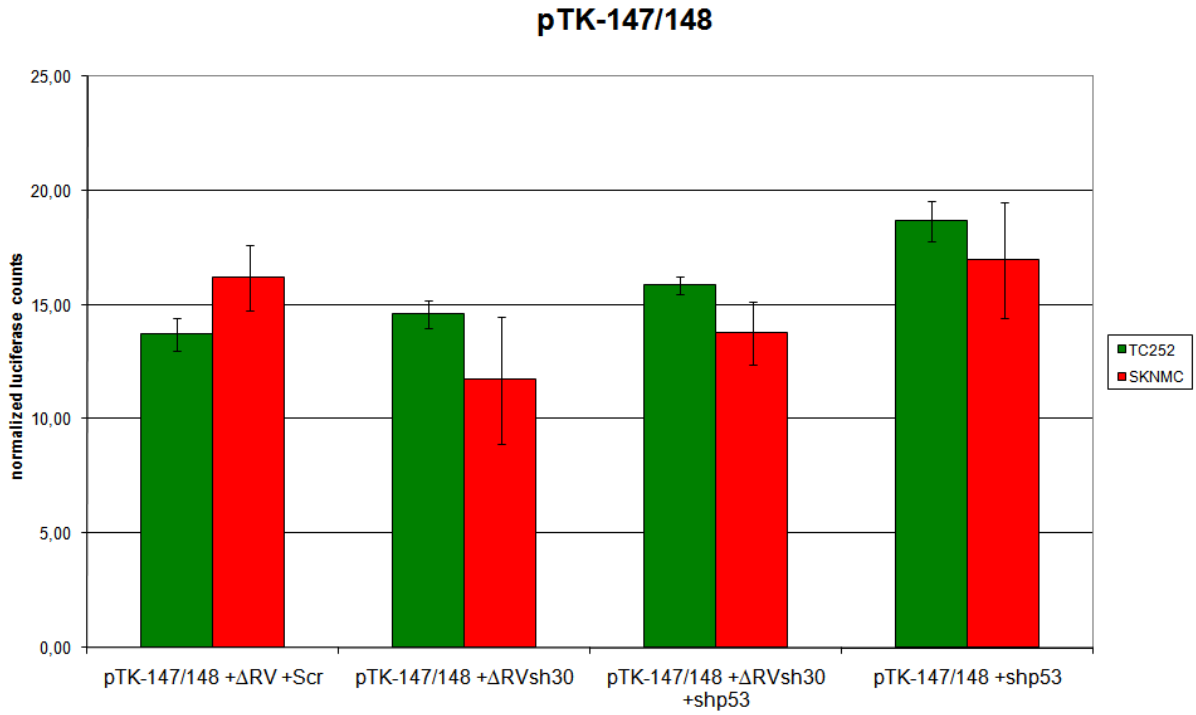

Fig. 16: Activation/Repression of the p21-construct pTK-147/148 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30) and p53 (shp53). Transfections were done in sextuplicates.

pTK-147/148 does not show any significant effect neither in TC252 nor SKNMC.

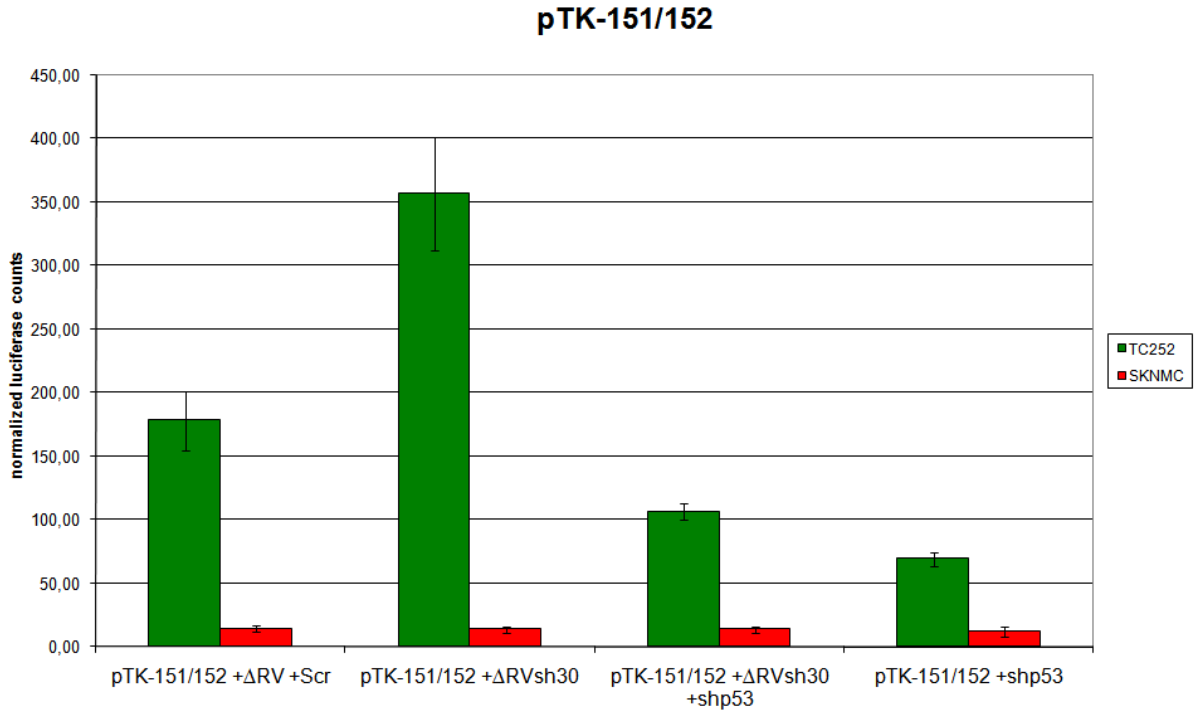

Fig. 17: Activation/Repression of p21-construct pTK-151/152 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30) and p53 (shp53). Transfections were done in sextuplicates.

The two fold induction effect upon knock down of EWS-FLI1, which was found in the last experiment, was recapitulared. Repression of p53 leads to a 2,6-fold decrease of p21-levels using fragment pTK-151/152, whereas knock down of EWS-FLI1 and p53 at the same time, result in a 3,5-fold reduction compared to EWS-FLI1 knock down alone and 1,6 fold reduction to the empty vector. The double knock down nearly shows the same results as the single p53 knock down, indicating that p53 is the main player. Additionally the p53 mutant Ewing Sarcoma cell line SKNMC does not show any effect.

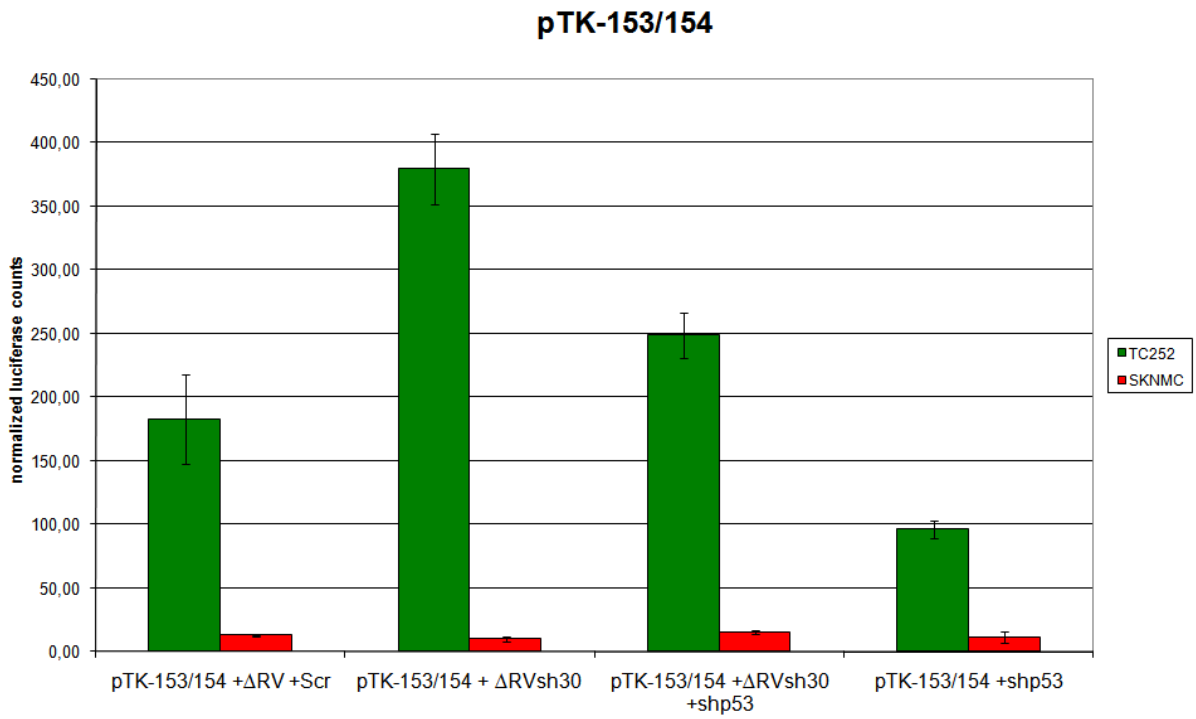

Fig. 18: Activation/Repression of p21-construct pTK-153/154 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30) and p53 (shp53). Transfections were done in sextuplicates.

Unfortunately the EWS-FLI1 knock down was not as effective as in the last experiment. pTK-153/154 shows now only a twofold induction when EWS-FLI1 is repressed. Nevertheless p21 levels are decreased by 50% when p53 is repressed compared to ΔRV and shScrambled +pTK-153/154 in TC252 and 25% when both EWS-FLI1 and p53 are knocked down compared to the single knock down of EWS-FLI1. Again there are no effects visible in SKNMC cells.

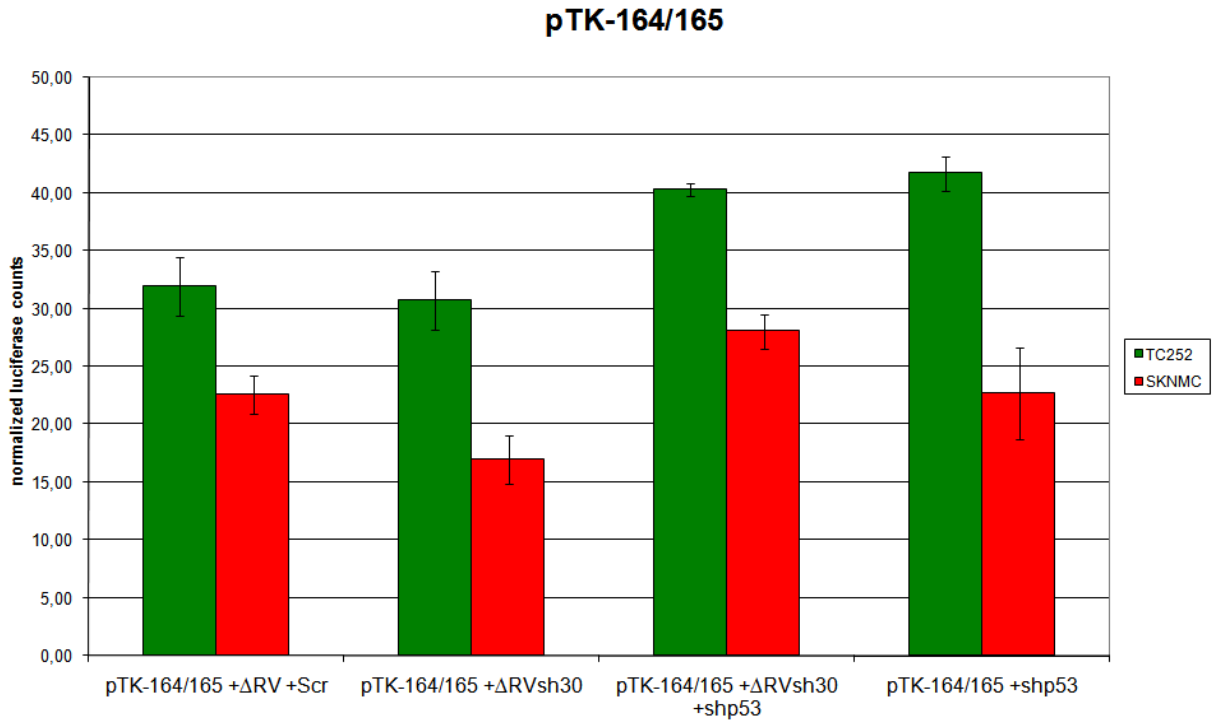

Fig. 19: Activation/Repression p21-construct pTK-164/165 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30) and p53 (shp53). Transfections were done in sextuplicates.

pTK-164/165 does not show any notable effect neither in TC252 nor SKNMC.

3.1.4 Future prospects

Site directed mutagenesis and deletions of transcription factor binding sites have to be performed and of course tested in reporter gene assays in the presence and absence of EWS-FLI1, p53 and induced p53. Using the QuikChange® II Site-Directed Mutagenesis Kit (see also „Material and Methods“) the p53 and Ets1 binding sites in pTK-151/152 and pTK-153/154 should be mutated. To study the involvement of the Notch pathway in the induction of p21, the Cbf1 binding site in pTK-151/152 should be deleted. Additionally all possible combinations of transcription factor binding site mutations should be generated. So far, both the Ets1 and the p53 binding site in pTK-151/152 were successfully mutated, as well as two p53 binding sites in pTK-153/154. The Cbf1 binding site in pTK-151/152 was already successfully deleted. These fragments should also be tested for their response to p53. Since most experiments were mainly done only once, they should all be repeated and protein extracts for Western Blots (Fli1, p53, p21 and β-actin) should be conducted. In the experiments presented in this Diploma thesis, reporter gene assays were performed without a selection step in order not to introduce additional stress to the cells.

Western blots, however, require an enrichment step for transfected cell (i.e. Puromycin selection).

As a control p21-plasmids should be transfected into a non-Ewing Sarcoma cell line were EWS-FLI1 should be co transfected and expressed, so far these attempts have failed due to difficulties in finding an appropriate human p53 wild type cell line.

3.2 Jagged1

To gain a better understanding of the regulation of Jagged1, a 573bp of the Jagged1 promoter was cloned into the luciferase reporter vector pGL4.10 via BglII/HindIII and fused to an already existing 1,2kb Jagged1 containing plasmid lacking the actual promoter sequence. Both plasmids were then analyzed in gene reporter assays in the presence and absence of EWS-FLI1. A time course, where reporter gene assays were done 48, 72 and 96h after transfection, revealed that 96h shows the best result, which is in line with our previous finding that Jagged1 expression upregulation by EWS-FLI1 knock down is rather a late event. [74]

3.2.1 Jagged1 reporter gene assays

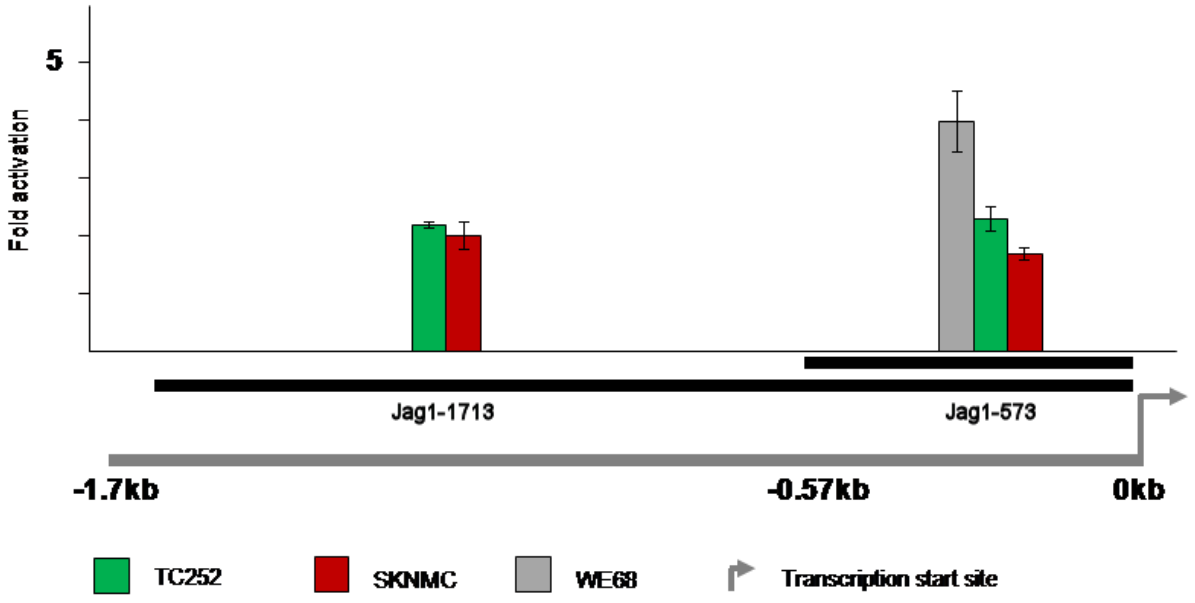

Fig. 20: Activation of Jagged1 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30). JAG1- 1713 was tested only once in triplicates in TC252 and SKNMC cells. JAG1-573 was tested three times in WE68, five times in SKNMC and seven times in TC252.

This assay was repeated several times, with and without Puromycin selection, at the beginning in triplicates, later in sextuplicates showing always around a twofold

induction when EWS-FLI1 was knocked down in TC252 and rather similar effects in SKNMC. On average a 2,3 fold increase in TC252 and 1,7 fold increase in SKNMC for the 573bp fragment, as well as a 2,2 fold induction in TC252 and a 2 fold induction in SKNMC for the 1,7kb fragment was observed. This is indicating that the responsive element is mostly within the first 573bp fragment. The smaller fragment was as well tested in the p53 wildtype Ewing's sarcoma cell line WE68. An average of three independent experiments revealed in a 4fold induction.

3.2.2 Future prospects

Both Jagged1 containing plasmids should be tested in ASP14 cells harboring a doxicyclin inducible EWS-FLI1 shRNA and in a non-Ewing Sarcoma cell line to establish the kinetics and ESW-FLI1 dependence of JAG1 promoter activity.

3.3 Hey1

The third target of our repression studies was Hey1, since the needed plasmids were already available in our lab, no cloning steps were involved.

In a first step both plasmids, Hey1-FL containing 3.9kb of Hey1 promoter region and Hey1-M5 containing 1.7kb were tested in reporter gene assays in the presence and absence of EWS-FLI1. All Hey1 experiments were done in sextuplicates without puromycin selection. The 3.9kb and 1.7kb containing plasmids showed an upregulation upon knock down of EWS-FLI1.

Knock down of EWS-FLI1 using shRNA showed in five different experiments an induction of Hey1-FL in TC252 and STAET-1, however, with high variability. In TC252 the induction was between 2 and 5,5fold and in STAET-1 between 1,5 and 5 fold. In Three independent experiments Hey1-M5 showed an induction between 1,4 and 2,7fold in STAET1. In TC252 the induction upon knock down of EWS-FLI1 for Hey1-1.7 was between 2 and 2,4fold.

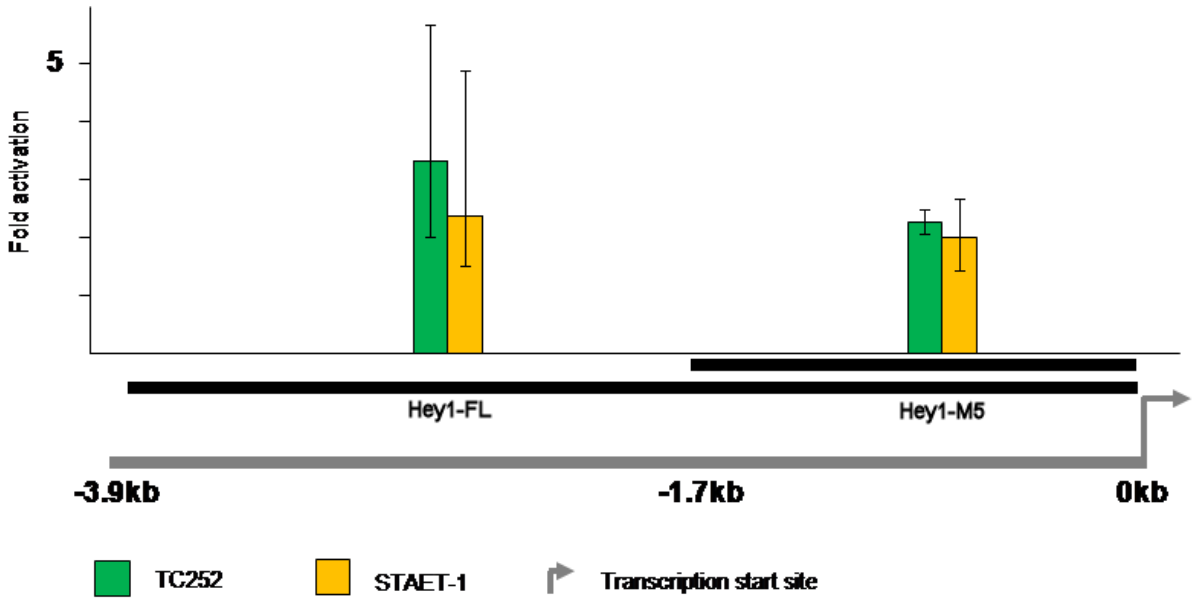

Fig. 21: Activation of Hey1-FL and Hey1-M5 upon shRNA mediated knock down of EWS-FLI1 (ΔRVsh30). Hey1-FL was tested five times in TC252 and STAET1 in sextuplicates. Hey1-M5 was tested three times in TC252 and STAET1 in sextuplicates.

Within the experiments mentioned above, the effect of γ-sectretase inhibitor (GSI) and numb was tested to block notch activation, which would be predicted to result in a down regulation of Hey1. So far, we saw some effect of GSI treatment, which was, however, not reproducible due to GSI toxicity to transfected cells. Since untransfected cells tolerate GSI very well, and no puromycin selection, to enrich for transfected cells, was performed, it is possible that the fraction of cells surviving until 72h after GSI treatment contains only untransfected cells. Preliminary experiments using ectopic numb expression resulted in an increase in Hey1 promoter activity.

However, since results varied significantly between experiments, they should be repeated under Puromycin selection, and Western Blots for EWS-FLI1 and β-actin as well as RT-PCR for EWS-FLI1, Hey1 and β-actin should be performed in the future.

4 Discussion

In an attempt to study transcriptionally activated target genes of the EWS-FLI1 fusion gene transcript, nearly as much transcriptional repressed targets were discovered. Among them was p21, a cell cycle inhibitor leading to cell cycle arrest in a p53 dependent manner.

10kb of the p21 promoter region were amplified in 14 overlapping fragments and cloned into a reporter gene vector. However, both the use of genomic DNA as well as a commercial available cosmid clone resulted in multiple sequence deviations from the publically available reference databases. Some of these deviations were reproducibly found in multiple clones of given constructs. We therefore wondered if these might be either mutations, polymorphisms or sequencing errors in the reference sequence. To address this question we performed a multiple sequence alignment of two NCBI human reference sequences, one from February 2007 and one from January 2008, the Celera reference sequence, the chimpanzee reference sequences and the corresponding p21 construct sequence. Comparison of the reference sequences showed that the actual NCBI sequence is 99% identical to the older version and the Celera sequence, as well as 98% to the chimpanzee sequences, leading to a high variability within the reference sequences and a rather complex analysis of the found deviations. We therefore decided to consider a mismatch a mutation, if the corresponding nucleotide in a p21 construct varied from all reference sequences. A single identity with the chimpanzee is most likely a mutation, but could also be evolutionary conserved. If at least one human reference sequence showed the same nucleotide than the p21 construct, we decided to assume it as wildtype. Nevertheless all variations were tested for loss or gain of transcription factor binding sites, using Genomatix. We took a matrix similarity score of 0,95 or higher as a threshold. Since no high score TFBS were lost or gained, we decided to use these constructs, even in the case of pTK-174/175 which had several mutations because of the difficulties of cloning it. Only the use of Sure2 Supercompetent cells enabled us to clone this fragment.

An in silico transcription factor binding site analysis of the 14 different p21 constructs, revealed that among them were some containing Ets binding sites, a few with p53 binding sites and only two of them contained both, Ets and p53 binding sites. To determine the involvement of p53 in p21 gene expression regulation by EWS-FLI1,

we performed reporter gene assays with all 14 fragments in a p53 wildtype and a p53 mutant Ewings's sarcoma cell line in the presence and absence of EWS-FLI1. Knock down of EWS-FLI1 in the p53 wildtype Ewing's sarcoma cell line TC252, resulted in very little to no induction in the majority of the 14 different p21-constructs tested. pTK-160/161 and pTK-170/171 contain Ets binding sites, but show nearly no induction of p21 upon the knock down of EWS-FLI1. This findings indicated that the binding of EWS-FLI1 to its transcription factor binding site is not sufficient to repress p21 and that some other mechanism or factor has to be involved, or that these TFBS are not essential for the regulation of p21 and EWS-FLI1 does not bind at all to these sites.. Since pTK-153/154 and pTK-151/152, which contain not only Ets binding sites but as well p53 binding sites, showed an induction of p21, we strongly suppose that p53 is involved in the gene expression regulation of p21 by EWS-FLI1. Another strong evidence was, that none of the 14 different fragments showed an effect in the p53 mutant cell line SKNMC. This finding indicated again, that wildtype p53 is essential for the regulation of p21 by EWS-FLI1. To further test our hypothesis we performed reporter gene assays with all 14 fragments in TC252 and SKNMC cells in the presence and absence of EWS-FLI1, but at the same time induced p53 via Etoposide treatment. Again 12 fragments showed very little to no induction in TC252 cells, whereas pTK-151/152 and pTK-153/154 were even more induced as with the knock down of EWS-FLI1 alone. In the case of pTK-153/154, etoposide treatment raised the induction of the reporter activity upon EWS-FLI1 knock down from fourfold to sevenfold. For pTK-151/152 the induction changed from 2,3 fold with shEWS-FLI1 only to a fourfold induction of the p21 promoter when p53 was induced with EWS-FLI1 silencing. The fact that 12 constructs, which contain different parts of the p21 promoter region, most of them containing either a potential p53 or an Ets binding site, do not react at all upon a down regulation of EWS-FLI1 and etoposide treatment, whereas those two which carry both Ets and p53 binding sites together showed high induction levels in a wildtype p53 background, strongly suggests that p53 is involved in the regulation of p21 by EWS-FLI1. One explanation might be that EWS-FLI1 competes with p53 for binding to the regulatory element on the DNA and therefore keeps p53 from binding to its TFBS thereby preventing p53 from activating p21. Another possibility would be, that both EWS-FLI1 and p53 are able to bind, but EWS-FLI1 binds as well to the activation domain of p53 thereby repressing it. The

hypothesis that p53 is involved, is validated by gene reporter assays in the p53 mutant cell line SKNMC, which showed no induction of p21 in all 14 constructs.

In a next experiment we performed gene reporter assays in the presence and absence of EWS-FLI1 in TC252 and SKNMC, but as well in the presence and absence of p53. Here we tested only four constructs, pTK-151/152 and pTK-153/154 due to their high induction upon knock down of EWS-FLI1, pTK-164/165 because it showed a slight upregulation in the absence of EWS-FLI1 and contains one p53 binding site, and pTK-147/148 which showed as well only a slight upregulation upon knock down of EWS-FLI1, but has neither an Ets binding site nor a p53 binding site. Both pTK-147/148 and pTK-164/165 showed no significant effect upon knockdown of either EWS-FLI1, p53 or of EWS-FLI1 and p53 together in both cell lines. In contrast in TC252 pTK-151/152 was twofold induced in the absence of EWS-FLI1, and a significant reduction in p21 levels upon the double knock down of EWS-FLI1 and p53, was observed. The single knock down of p53 nearly showed the same results as the double knock down, indicating that p53 expression is limiting for p21 promoter induction. The lack of any effect in the mutant p53 cell line SKNMC confirmed the involvement of p53 in the regulation of p21 by EWS-FLI1. In TC252 cells pTK-153/154 shows a reduction upon down regulation of p53 and EWS-FLI1, but an even stronger reduction upon down regulation of p53 alone. This result further supports the hypothesis that p53 is involved in the regulation of p21 gene expression by EWS-FLI1, especially since the mutant p53 cell line SKNMC showed no effect.

To sum up, p21 is induced in wildtype p53 Ewing's sarcoma cell line TC252 upon knockdown of EWS-FLI1, whereas it is not in the mutant p53 cell line SKNMC. Induction of p53 resulted in even higher p21 levels whereas down regulation of p53 reduced p21 levels in TC252, again no effect was visible in SKNMC. This activity was exclusively seen in the two fragments, which contain both Ets and p53 binding sites together. Findings of our lab support the present reporter gene assay results and indicate as well that p53 is involved in p21 expression regulation by EWS-FLI1 [74].

The mechanism how EWS-FLI1 modulates p53 and thereby represses p21 expression is not solved yet, but due to the fact that the transcription factor binding site for EWS-FLI1 and p53 in the p21 promoter are very close to each other (in pTK-151/152 only 10bp apart) a physical interaction between EWS-FLI1 and the activation domain of p53 is possible, or they might compete for binding to DNA.

Another possible mechanism would be that EWS-FLI1 represses p53 and thereby down regulates p21, leading to the question how EWS-FLI1 represses p53. This might be either directly or via other factors. One possibility would be via the Notch pathway and will be discussed in the following.

Affymetrix array data revealed that the Notch ligand Jagged1 is down regulated in several Ewing's sarcoma cell lines. To study the transcriptional repression of Jagged1, we tested a 573bp and a 1713bp fragment of the Jagged1 promoter in gene reporter assays. We found an upregulation of Jagged1 in wildtype p53 Ewing's sarcoma cell line TC252, as well in the p53 mutant cell line SKNMC in the absence of EWS-FLI1. The 573bp construct showed a 2,3fold increase in TC252, a 1,7 fold increase in SKNMC, and a 4fold increase in WE68 cells upon knock down of EWS-FLI1. The 1713bp construct showed a similar induction, 2,2 fold in TC252 and 2 fold in SKNMC, indicating that the regulatory element responsible for the repression of Jagged1 by EWS-FLI1 is within the first 573bp. Since only a single, and low score Ets transcription factor binding site is present in this sequence it is unlikely that EWS-FLI1 directly represses Jagged1.

Recently our lab demonstrated that the knockdown of EWS-FLI1 resulted in upregulation of endogenous Jagged1, resulting in activation of the Notch pathway and consequently in an increase of Hey1, which down modulated MDMX and led to p53 accumulation [74]. Interestingly also Hey1 was found to be repressed by EWS-FLI1 and gene reporter assays using a 3.9kb and 1.7kb promoter containing construct, revealed an induction of Hey1 upon knock down of EWS-FLI1. Although an induction was found in each experiment, the variation was quite high. In TC252 the induction was between 2 and 5,5fold and in STAET-1 between 1,6 and 5 fold for the 3.9kb containing construct. The 1.7kb containing plasmid showed an induction between 1,4 and 2,7fold in STAET1 and in TC252 between 2 and 2,4fold. Due to the high variance the numbers are difficult to interpret, but one could suppose that the regulatory element involved in the repression of Hey1 by EWS-FLI1 is more likely outside the first 1.7kb of the promoter since induction levels with the 3.9kb fragment were higher. Most Ets binding sites and those with the highest scores are located in the sequence between 1.7kb and 3.9kb and therefore seem to be of special interest, as well as four SMAD binding sites in the same region.

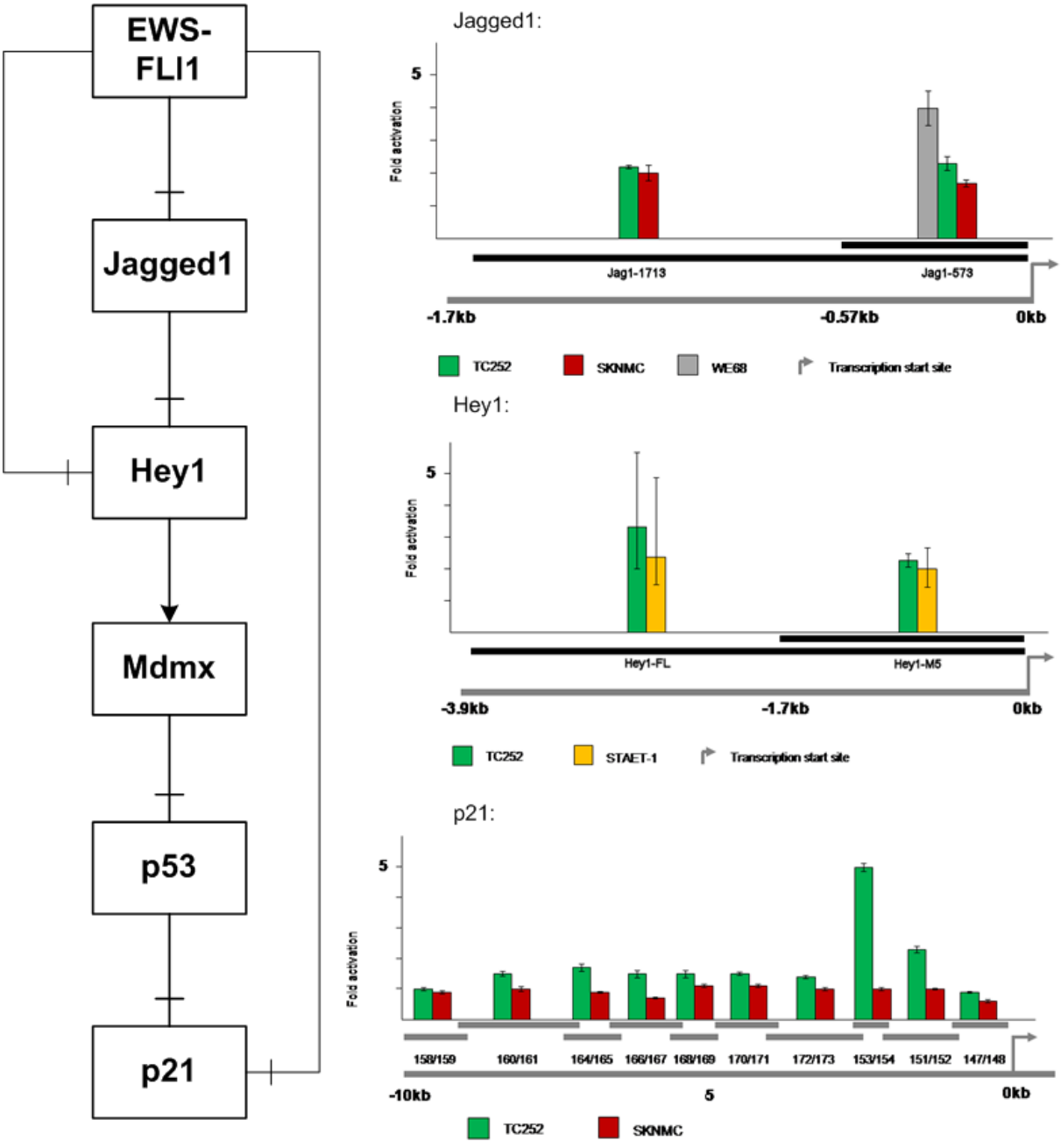

Fig. 22: Repression of p21, Jagged1 and Hey1 by EWS-FLI1

The actual mechanisms are still not clear and need further investigation, but it appears that Jagged1 is indirectly repressed by EWS-FLI1, blocking the Notch pathway, which regulates the expression of Hey1. In addition Hey1 itself may directly be repressed by EWS-FLI1. When Hey1 gets activated it induces p53 via down regulation of MDMX, leading to activation of p21. Therefore regulation of p21 expression in Ewing Sarcomas may not only be the result of a direct interaction of EWS-FLI1 with p21 or p53, but EWS-FLI1 may as well regulate p53 and thereby p21 via the Notch pathway.

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Appendix

p21-reporter gene constructs:

pTK-146/147:

CTGTGAAATAAACGGGACTGAAAAATCATTCTGGCCTCAAGATGCTTTGTTGGGGTGTCTAGGTGCTCCAGGTGC
TTCTGGGAGAGGTGACCTAGTGAGGGATCAGTGGGAATAGAGGTGATATTGTGGGGCTTTTCTGGAAATTGCAGA
GAGGTGCATCGTTTTTATAATTTATGAATTTTTATGTATTAATGTCATCCTCCTGATCTTTTCAGCTGCATTGGG
TAAATCCTTGCCTGCCAGAGTGGGTCAGCGGTGAGCCAGAAAGGGGGCTCATTCTAACAGTGCTGTGTCCTCCTG
GAGAGTGCCAACTCATTCTCCAAGTAAAAAAAGCCAGATTTGTGGCTCACTTCGTGGGGAAATGTGTCCAGCGCA
CCAACGCAGGCGAGGGACTGGGGGAGGAGGGAAGTGCCCTCCTGCAGCACGCGAGGTTCCGGGACCGGCTGGCCT
GCTGGAACTCGGCCAGGCTCAGCTGGCTCGGCGCTGGGCAGCCAGGAGCCTGGGCCCCGGGGAGGGCGGTCCCGG
GCGGCGCGGTGGGCCGAGCGCGGGTCCCGCCTCCTTGAGGCGGGCCCGGGCGGGGCGGTTGTATATCAGGGCCGC
GCTG

pTK-147/148:

CTGGGAGCGGATAGACACATCACTCATTTCTGTGTCTGTCAGAAGAACCAGTAGACACTTCCAGAATTGTCCTTT
ATTTATGTCATCTCCATAAACCATCTGCAAATGAGGGTTATTTGGCATTTTTGTCATTTTGGA**G**CCACAGAAATA
AAGGATGACAAGCAGAGAGCCC**C**GGGCAGGAGGCAAAAGTCCTGTGTTCCAACTATAGTCATTTCTTTGCTGCAT
GATCTGAGTTAGGTCACCAGACTTCTCTGAGCCCC**C**GTTTCCCCAGCAGTGTATACGGGCTATGTGGGGAGTATT
CAGGAGACAGACAACTCACTCGTCAAATCCTCCCCTTCCTGGCCAACAAAGCTGCTGCAACCACAGGG**A**TTTCTT
CTGTTCAGGTGAGTGTAGGGTGTAGGGAGATTGGTTCAATGTCCAATTCTTCTGTTTCCCTGGAGATCAGGTTGC
CCTTTTTTGGTAGTCTCTCCAATTCCCTCCTTCCCGGAAGCATGTGACAATCAACAACTTTGTATACTTAAGTTC
AGTGGACCTCAATTTCCTCATCTGTGAAATAAACGGGACTGAAAAATCATTCTGGCCTCAAGATGCTTTGTTGGG
GTGTCTAGGTGCTCCAGGTGCTTCTGGGAGAGGTGACCTAGTGAGGGATCAGTGGGAATAGAGGTGATATTGTGG
GGCTTTTCTGGAAATTGCAGAGAGGTGCATCGTTTTTATAATTTATGAATTTTTATGTATTAATGTCATCCTCCT
GATCTTTTCAGCTGCATTGGGTAAATCCTTGCCTGCCAGAGTGGGTCAGCGGTGAGCCAGAAAGGGGGCTCATTC
TAACAGTGCTGTGTCCTCCTGGAGAGTGCCAACTCATTCTCCAAGTAAAAAAAGCCAGATTTGTGGCTCACTTCG
TGGGGAAATGTGTCCAGCGCACCAACGCAG

Bp 261 of pTK-147/148: C

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: A

Bp 369 of pTK-147/148: A

NCBI-human 01.08: A

NCBI-human 02.07: G

Celera: A

Chimp sequence: G

pTK-149/150:

CATCTGCAAATGAGGGTTATTTGGCATTTTTGTCATTTTGGA**A**CCACAGAAATAAAGGATGACAAGCAGAGAGCC
CCGGGCAGGAGGCAAAAGTCCTGTGTTCCAACTATAGTCATTTCTTTGCTGCATGATCTGAGTTAGGTCACCAGA
CTTCTCTGAGCCCCAGTTTCCCCAGCAGTGTATACGGGCTATGTGGGGAGTATTCAGGAGACAGACAACTCACTC
GTCAAATCCTCCCCTTCCTGGCCAACAAAGCTGCTGCAACCACAGGG**G**TTTCTTCTGTTCAGGTGAGTGTAGGGT
GTAGGGAGATTGGTTCAATGTCCAATTCTTCTGTTTCCCTGGAGATCAGGTTGCCCTTTTTTGGTAGTCTCTCCA
ATTCCCTCCTTCCCGGAAGCATGTGACAATCAACAACTTTGTATACTTAAGTTCAGTGGACCTCAATTTCCTCAT
CTGTGAAATAAACGGGACTGAAAAATCATTCTGGCCTCAAGATGCTTTGTTGGGGTGTCTAGGTGCTCCAGGTGC
TTCTGGGAGAGGTGACCTAGTGAGGGATCAG

Bp 43 of pTK-149/150: A

NCBI-human 01.08: G

NCBI-human 02.07: A

Celera: G

Chimp sequence: G

Bp 273 of pTK-149/150: G

NCBI-human 01.08: A

NCBI-human 02.07: G

Celera: A

Chimp sequence: G

pTK-151/152:

CAGGTAAACCTTAGCCTGTTACTCTGAACAGGGTATGTGAT**A**TGCCAGCAGATCCTTGCGACAGGGCTGGGATCT
GATGCATGTGTGCTTGTGTGAGTGTGTGCTGGGAGTCAGATTCTGTGTGTGACTTTTAACAGCCTGCTCCCTTGC
CTT**C**TTCAGGGCAGA**G**GTCCTCCCTTAGAGTGTGTCTGGGTACACATTCAAGTGCATGGTTGCAAACTTTTTTTT
TTAAAGCACTGAATAGTACTAGACACTTAGTAGGTACTTAAGAAATATTGAATGTCGTGGTGGTGGTGAGCTAGA
AGTTATAAAAAAAATTCTTTCCCAAAAACAACAACAAAAAGAATTATTTCATTGTGAAGCTCAGTACCACAAAAA

TTTAAATAATTCATTACAAGCCTTTATTAAAAAAAATTTTCTCCCCAAAGTAAACAGACAGACAATGTCTAGTCT
ATTTGAAATGCCTGAAAGCAGAGGGGCTTCAAGGCAGTGGGAGAAGGTGCCTGTCCTCTGCTGGACATTTGACAA
CCAGCCCTTTGGATGGTTTGGATGTATAGGAGCGAAGGTGCAGACAGCAGTGGGGCTTAGAGTGGGGTCCTGAGG
CTGTGCCGTGGCCTTTCTGGGGTTTAGCCACAATCCTGGCCTGACTCCAGGGCGAGGCAGGCCAAGGGGGTCTGC
TACTGTGTCCTCCCACCCCTACCTGGGCTCCCATCCCCACAGCAGAGGAGAAAGAAGCCTGTCCTCCCCGAGGTC
AGCTGCGTTAGAGGAAGAAGACTGGGCATGTCTGGGCAGAGATTTCCAGACTCTGAGCAGCCTGAGATGTCAGTA
ATTGTAGCTGCTCCAAGCCTGGGTTCTGTTTTT**C**AGTGGGATTTCTGTTCAGATGAACAATCCATCCTCTGCAAT
TTTTTAAAAGCAAAACTGCAAATGTTTCAGGCACAGAAAGGAGGCAAAGGTGAAGTCCAGGGGAGGTCAGGGGTG
TGAGGTAGATGGGAGCGGATAGACACATCACTCATTTCTGTGTCTGTCAGAAGAACCAGTAGACACTTCCAGAAT
TGTCCTTTATTTATGTCATCTCCATAAACCATCTGCAAATGAGGGTTATTTGGCATTTTTGTCATTTTGGAGCCA
CAGAAATAAAGGATGACAAGCAGAGAGCCCCGGGCAGGAGGCAAAAGTCCTGTGTTCCAACTATAGTCATTTCTT
TGCTGCATGATCTGAGTTAGGTCACCAGACTTCTCTGAGCCCC**C**GTTTCCCCAGCAGTGTATACGGGCTATGTGG
GGAGTAG

Bp 42 of pTK-151/152:		A
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	C
Bp 154 of pTK-151/152:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	C
	Celera:	T
	Chimp sequence:	C
Bp 166 of pTK-151/152:		G
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A
	Celera:	A
	Chimp sequence:	G
Bp 859 of pTK-151/152:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	C

Celera: T

Chimp sequence: T

Bp 1244 of pTK-151/152: C

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: A

pTK-153/154:

TGCCTCTGCTCAATAATGTTCTATCTTTGTTCCGCCTCTTCTCTGGGGTCTCACTTCTTGGGAGCCTGTGTGAAG
GTGAATTCCTCTGAAAGCTGACTGCCCCTATTTGGGACTCCCCAGTCTCTTTCTGAGAAATGGTGACATTGTTCC
CAGCACTTCCTCTCCCTTCCTAGGCAGCTTCTGCAGCCACCACTGAGCCTTCCTCACATCCTCCTTCTTCAGGCT
TGGGCTTTCCACCTTTCACCATTCCCCTACCCCATGCTGCTCCACCGCACTCTGGGGAGGGGGCTGGACTGGGCA
CTCTTGTCCCCCAGGCTGAGCCTCCCTCCATCCCTATGCTGCCTGCTTCCCAGGAACATGCTTGGGCAGCAGGCT
GTGGCTCTGATTGGCTTTCTGGCCGTCAGGAACATGTCCCAACATGTTGAGCTCTGGCATAGAAGAGGCTGGTGG
CTATTTTGTCCTTGGGCTGCCTGTTTTCAGGTGAGGAAGGGGATGGTAGGAGACAGGAGACCTCTAAAGACCCCA
GGTAAACCTTAGCCTGTTACTCTGAACAGGGTATGTGATCTGCCAGCAGATCCTTGCGACAGGGCTGGGATCTGA
TGCATGTGTGCTTGTGTGAGTGTGTGCTGGGAGTCA

pTK-158/159:

CGAACGTGGGTGGGAGATGATGCCAGTCCCTTCTGAGCCTGGTGTGAAGAAATGCACGTGGCTTCTTTGGCACTT
TCCCCTACCTCCAGCTGCAGTCAGAGATCATGAGGCCCCAGGGAATGGTGGAGCCACATGGTGGAAAGAACCTAA
GAACCTGAATCACCACACGGAGGGCAGCCACCGGCCCACCAGAACACCTCTATTGGATTCATATGTGAGTGAGAA
ATAAACTTGTATTTGGCTTGTCACTGAAATGTGAGAGTTTATTTATTACAGGAGCTAGTGTCGTCCTATCACTCA
CCCTGACTTTTCTTCATAAGTCCAGTCCTAAGCTGCTAGGATGGGCGTAGGAGGAGACATCAGCAATGATTCCTC
CCAGCAGATACAGGGTTGTTAGAAACCACTGAGGATAGGGAAGAGGGAGTGACATCTCCTCCCTGGCTCTGAACT
TGGCTCTAGTGTGGCCACTAGCATTTGGGGCTTGGGGGTTGCAGCTGTCGCATTCCACAGTGGCCCCGATGGCAT
TACAATTACAGATGACACTTAGAGCCACCCTAGGGAAGCAAAGGCAAGGACAAGCAGCTGATGGGAGGGGGAGCA
GAAGGGCTGCGGCCACAGCCAGAGCCCCGGATCTGCCCTGACCCTGGTCTGTGTCCCCTTAGCCGAAGCCAGCAC
TTCCTAGGGTCTCCATTTCTCTGTCTGTATAAGCGTCTAATACCCCTGCCCTGGTTGGGGGAGGGAGGAGCATGT
GGATGGTGAGGAGTGAGAGAATGGATCTGAAACCTGATGTCCCTGAGATTCCACACTGCTGTGCAATCAGAGGCC
CCGAACCCAAGCCCCTCAGCCTATGCTAGGCTTTTTCTTGGGAGGGGTTTAAAAAATATGTTAGGGAAGAAAAAA
AAATCAAAGTGCCCAATTCCTATTATTTTGGGATCATCCAGAAGGCTGTTGTGAGCTAAGGGACCTGGAGGGGGT
GTCTTGGGAGCTTTGAAGGCAGCTCACAGGACTGAATTCAGAATCTGTGGGCAGCTCCCTTTGCTGCCAAATGTC
TATTCTCAGGTGTCACAATCGTTGAG

pTK-160/161:

CGACCTGGAGGGGGTGTCTTGGGAGCTTTGAAGGCAGCTCACAGGACTGAATTCAGAATCTGTGGGCAGCTCCCT
TTGCTGCCAAATGTCTATTCTCAGGTGTCACAATCGTTGAAATTCTTCCATTAGGGCTTCATTCCTTTGGATTAC
TTTTTGTTTCTA**C**ACAAAAATGCAAGCAATGATAATCCCCAATATGTGTTCAGGGCTTTAGACTTGTTCCAATAA
TCCTAAAAGACACAGACAGACAGTGAGGCCCAGATACCTGGTGTGGCTACTTCTTCAATCATGCTTCTCCTGACA
GAGTGATTATCGGGCAACTCGACTCCTAGGTATATAGCCAGAAACTCTTGCACATGTGCCCTAGAAACACGGATA
AAAATACTTATAGTAGCCCTATGTGTAATAACAAAAATTGGCGATAACCCAAACCCATTGATAGGAGAAGGAATA
ATTGGATATTTAAAAAACTGAAAATGGGCCGGGCATGGTGGCTCATGCCTGTAATCCCAAGACTTTGGGAGGTTG
AGGCTGAAGTTTGAGCTCAGGTGTTTGAGACCGCCTGGGTAACATAGGGAGACACCACCTCTATTTAAAAAAAAA
AAATTAGCCAGGCTTCGTGGTGTGCACATGTGGTTCCAGCTACTTGGGAGCTGAGGTAGGAGGATTGCTTGAGGC
TGGGAGGTCAAGGCTGCAGTGAGCCATGATCACACCACTGTACTCCAGCCTGGGCGGCAGATTGAGGCCCTGTCT
CAAAATAAATAAGTAAATAACATAGAAACCGGAAAACGATTGAATCATGTTTAAGTTACGCATTGCTGCCTAAAA
AATCATGTTAAAACACAGTGGCAACCACGATCATTTGTTTGCTCACATTCTATAATTCAGTCGGGGTTTACTGGG
GATGGCTTGTCTCTACTTCATGTGGCTCCACCTGGGGAGGTTCAACTGGAGCTGGGGGATCAACTCTCAAAATGG
TCCTCCTATGGCTGGCAAGTTGATGCTGACTGTTTGCTGGGAGGTCTGCTGGAACTCTTAGCTAGTGGGCCTTGG
TTATCCTGCGTGTGACCTCTCCACAAGACTTCTCACAGCATGATGGTCTCAGAGTAATCAAACTTCTTACATGGT
GGCTTAAGGTCTCTAAGAATACAAAAGCAGTAGAAGTTACTATACCTTTTTAAGGCTTAGGTCTGGAATGGGCAC
AG**G**GTTACTTCTGTCCCATTCTATTGGCTAAAAGAGTCACAAGCCAGAAATGTTCA**A**GGGGAGGCAACTACAAAA
CAGTGTGAATACCCAGAGGTATACCTCACTGGGAGACATTGACTTCCACAAACCACAACTACCCACAACTACATG
GATAAATATTAGGTTAGGTTATGCTTCAGTATCAAATAACCTCTACATTTCAGTGACTTCACACACAAATGGTTA
CTTTTTTCTTATATACCTATCCCATAGAAGTTGGTGGGGCACTCTGCTCCACGCATTCACTCAGGGATCCAGGCT
GATGGAGGCTCCACCATGTTGGAGTGGCACCATTGCACCACCTGGGACATGAGACCTCACCTTTGCCACAACTGT
GAAGGACAGAAAGGCTCGAGAATTTCACCTGGCCTCAGCTCTAAAGAGACAAATGTCACTTATGCTCACATTTCA
TCAGCTAGGACTAGTCGCATGATCCCATCTACACTGTAAGTGGGGCTGGAAAATGTTGGAGAACACCTGGAAAAA
TGGCTGAGCATTACTGTCTTGGCCACATGTGATAGAACTATTTAAAACAAAAAAAGTTTTACATAAGATGCAAGG
TAGTGTTCACTTTGGGGTAGGGGTGGAACACACAAGAAAGTGTCAGTGATTTGTAATATTCTAGTTTTTGGTTTG
AGCAGTGAGTGTTAATTATTTTTTAAAACAAATGAATGAATGAATGGACAAATGAATAAATAAAAGAGAGGGCTC
GAGGCAGCAGCACTGAAGCCTAACCTCACAGTACAGGCCAAAAATGACCCTG

Bp 263 of pTK-160/161:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	T

Bp 1202 of pTK-160/161:		G
	NCBI-human 01.08:	G
	NCBI-human 02.07:	G

Celera: A

Chimp sequence: G

Bp 1257 of pTK-160/161: A

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: C

Chimp sequence: A

pTK-162/163:

CTATCCCATAGAAGTTGGTGGGGCACTCTGCTCCACGCATTCACTCAGGGATCCAGGCTGATGGAGGCTCCACCA
TGTTGGAGTGGCACCATTGCACCACCTGGGACATGAGACCTCACCTTTGCCACAACTGTGAAGGACAGAAAGGCT
CGAGAATTTCACCTGGCCTCAGCTCTAAAGAGACAAATGTCACTTATGCTCACATTTCATCAGCTAGGACTAGTC
GCATGATCCCATCTACACTGTAAGTGGGGCTGGAAAATGTTGGAGAACACCTGGAAAAATGGCTGAGCATTACTG
TCTTG

pTK-164/165:

CGGCTCGAGAATTTCACCTGGCCTCAGCTCTAAAGAGACAAATGTCACTTATGCTCACATTTCATCAGCTAGGAC
TAGTCGCATGATCCCATCTACACTGTAAGTGGGGCTGGAAAATGTTGGAGAACACCTGGAAAAATGGCTGAGCAT
TACTGTCTTGGCCACATGTGATAGAACTATTTAAAACAAAAAAAGTTTTACATAAGATGCAAGGTAGTGTTCACT
TTGGGGTAGGGGTGGAACACACAAGAAAGTGTCAGTGATTTGTAATATTCTAGTTTTTGGTTTGAGCAGTGAGTG
TTAATTATTTTTTAAAACAAATGAATGAATGAATGGACAAATGAATAAATAAAAGAGAGGGCTCGAGGCAGCAGC
ACTGAAGCCTAACCTCACAGTACAGGCCAAAAATGACCCTTCAATAAACATTCACTGAGTTCCCAGCTGAAGCTG
GTAGGCCTCTCCAAGGTACAAAGATGAATAAAATATGGGCTCAGCCCCTCTAAGTGCCCAGCTCAGAGGATTCAG
TAGACACATAAACCAAGCAGGAGGGCAAAGTACAGTGGAGAGTCTTGCTCAGTGGGAGCTCTGGGAG**C**AGAGGGA
GCCCCGAGTAGGCCTCACCCCAAGTCACATGTGTTGGGTTATGAAGAATCAATAGGAATTCAAAGGTGAAGAATG
GAGTGGAGGTGGGGCAAGTTGGTCAGGGACACTCATTTTACCAAGAGAGGAAGATTTGTGCAAAGGCACAAAGAG
GCCTTCACCCCTCAGGTGGCCTGTGTTCACAGCATTTCCTCACTGCCTTCTAGTCCCTAAATGATCTGATCAACT
TACTCCCTCTGTACTGTACCACCCAGAAGCTTCTGCAAGAGCCAGGCACTTGTCCTCCACAATGGCTGCCATGAG
TGTTGGTTGCCGATGGTTACAGCCAAGTCGTTCCTTGGCCTTAGAGG**C**GGAAGCTCTCTTGCCCGAGGG

Bp 593 of pTK-164/165: C

NCBI-human 01.08: T

NCBI-human 02.07: T

Celera: T

Chimp sequence: C

Bp 948 of pTK-164/165: C

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: A

pTK-166/167:

CTCAGGTGGCCTGTGTTCACAGCATTTCCTCACTGCCTTCTAGTCCCTAAATGATCTGATCAACTTACTCCCTCT
GTACTGTACCACCCAGAAGCTTCTGCAAGAGCCAGGCACTTGTCCTCCACAATGGCTGCCATGAGTGTTGGTTGC
CGATGGTTACAGCCAAGTCGTTCCTTGGCCTTAGAGG**C**GGAAGCTCTCTTGCCCGAGGGCATGCACTCCCCTACT
GCAGGGGCAGCCACGTTTAGATGACTGTTTGGAAACCCAGGGTGTAAGGGCCTAGCTCTCATGCTTCAATTCAGC
GAACTCTGCAGGACCATCCCAGCTCCAGGGCTCCTGGTGGGATCAGCCAAG**G**CCTCTGTGGGCCTCCAGCCTCCA
T**T**AGTTCCCGGTTCTTCTCTCTGCCCAATTCC**A**TTTCTTTTATATCCAGACAAGTGTTGATTGTGAATGCACTCC
CCAATAAACT**T**CCTTCAAGCAAAACTCTATGTCAGAATGTG**T**TTTCTGGGAAACCTACCTAAGATACCCACTAAA
ATATAGATAATTACTAAATTCCAAGGGATGCCTTTCCTGGGGATGTTTGTACAGCAATTATC**C**TCATAACTAATA
TTCATGGAGTGATTTCTATGTGCCAAGCTAAGCACTTTGCTTTTAAGATCATATTTAATGAAGAACAGAGCCTCA
CAGTAAGAATACATGTCTTTTCTTTTA**T**TTATTTATTTATTTATTT**ATTTATTTATTTT**GAGACAGAGTTTTGCT
CTTGTTGCCCAGGCTGGAGTGCAATGGCGCAATCTCAGCTTACTGCAACCTCCGCCTCCCGAGTTCAAGTGATTC
TCCTGCCTCAGCCTCCCAAGTAGCTGGGATTACAGGCATGCACCACCATGTCCAGCTAATTTTGTATTTTTAGTT
ATAGATTTCTTTGCTCCTTCTCCACTCCCACTGCTTCATTTAACTAGCCTTAAAAAAATTATTAAAAATAAAAAT
AAATAGGCCTGGCCAGGCGTGGTGGTTCGTGCCTGTAATCCCAGCACTTGGGAGGCTGAGGCAGGCGGATCACCT
GAGTTCTGGACTTTGAGACCAGCCTGGCCAACATGGTGAAACCCGTCTGTACTAAAAACTATTAAAAAATTAGCC
AGGCATAGTGGCAGGAGCCTGTAATCCCAGCTACTTGGGAGGCTGGGGCAGGAGAATTGCTTGAACCTGGGAGGT
GGAGGTTGCAGTGAACTGATA**A**CGCACCATTGCATTCCG

Bp 188 of pTK-166/167: C

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: A

Bp 352 of pTK-166/167: G

NCBI-human 01.08: T

NCBI-human 02.07: T

Celera: T

Chimp sequence: G

Bp 377 of pTK-166/167:	T
NCBI-human 01.08:	C
NCBI-human 02.07:	C
Celera:	C
Chimp sequence:	C
Bp 408 of pTK-166/167:	A
NCBI-human 01.08:	G
NCBI-human 02.07:	G
Celera:	G
Chimp sequence:	G
Bp 461 of pTK-166/167:	T
NCBI-human 01.08:	C
NCBI-human 02.07:	C
Celera:	T
Chimp sequence:	T
Bp 492 of pTK-166/167:	T
NCBI-human 01.08:	A
NCBI-human 02.07:	A
Celera:	A
Chimp sequence:	T
Bp 588 of pTK-166/167:	C
NCBI-human 01.08:	A
NCBI-human 02.07:	A
Celera:	A
Chimp sequence:	A
Bp 703 of pTK-166/167:	T
NCBI-human 01.08:	C
NCBI-human 02.07:	C

	Celera:	T
	Chimp sequence:	T

Bp 722 to 734 of pTK-166/167:		ATTTATTTATTTT
	NCBI-human 01.08:	-
	NCBI-human 02.07:	-
	Celera:	-
	Chimp sequence:	-

Bp 901 of pTK-166/167:		A
	NCBI-human 01.08:	G
	NCBI-human 02.07:	G
	Celera:	G
	Chimp sequence:	A

Bp 1222 of pTK-166/167:		A
	NCBI-human 01.08:	T
	NCBI-human 02.07:	T
	Celera:	T
	Chimp sequence:	T

pTK-168/169:

CGCCAGGCGTGGTGGTTCGTGCCTGTAATCCCAGCACTTGGGAGGCTGAGGCAGGCGGATCACCTGAGTTCTGGA
CTTTGAGACCAGCCTGGCCAACATGGTGAAACCCGTCTGTACTAAAAACTATTAAAAAATTAGCCAGGCATAGTG
GCAGGAGCCTGTAATCCCAGCTACTTGGGAGGCTGGGGCAGGAGAATTGCTTGAACCTGGGAGGTGGAGGTTGCA
GTGAACTGATA**A**CGCACCATTGCATTCCGGCCTGGGCGACAGAGTGAAACTCCATCT**T**GAAAATAAATAAATAAA
TAAATAAATAAATAAATA**AATA**GGCCTGGCATGGTGGCTCACACCTGTAATCTCAGCACTTTGGGAGGCTGAGGC
AGCCAGATAATTT**G**AGGTCAGGAGTTCAAGACCAGCTTGGCTAACATGATGAAACATGACGAAACCCCCGTCTCT
GCTAAAAATTCAAAAATTAGCCAGTCATGGT**G**GTACATGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGGA
AAATCGCTTGAACCTAGAAAGTGGAGGTTGCAGTGAGCTGAGATCACGCCACTGCACTCCAGCTTGGGCAACAGA
GCAAGATTCCATCTCAAAAAATATATAAAAAATAAAAATAGGCTGGGCGCAGTGGCTCACGCCTGTAATCTCAGC
ACTTTGGGAGACCGAGGCAGACAGATCAACTGAGGTCGAGGTTTGAGACCAGCCTGACCAACATAGAGAAACCCT
CTCTCTACTAAAATAC

Bp 237 of pTK-168/169: A

NCBI-human 01.08: T

NCBI-human 02.07: T

Celera: T

Chimp sequence: T

Bp 283 of pTK-168/169: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

Bp 319 of 322 pTK-168/169: AATA

NCBI-human 01.08: -

NCBI-human 02.07: -

Celera: -

Chimp sequence: -

Bp 389 of pTK-168/169: G

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: G

Bp 482 of pTK-168/169: G

NCBI-human 01.08: T

NCBI-human 02.07: T

Celera: G

Chimp sequence: G

pTK-170/171:

CTGGGAGACCGAGGCAGACAGATCAACTGAGGTCGAGGTTTGAGACCAGCCTGACCAACATAGAGAAACCCTCTC
TCTACTAAAATACAAACATTGGGTGGGGCGAGTCATCGTCTGACGTCTGGCCGTGAGATGTTTCGGGAGCCGGGG
TCTCTCCGCTGCAGACATGACGAAGGGCCTTGTTTTAGGAATCTATTCCAAAGAAAAAGAAGATGATGTGCCACA

GTTCACAAGTGCAGGAGAGAATCTTGATAAATTGATAGCTGGAAAGCTGAGAGAGACTTTGAACATATCTGGACC

ACCTCTGAAGGCAGGCAAGACTCGAA**C**CTTTTATGGTCTGCATCAGGACTTCCCCAG**T**GTGGT**C**CTAGTTGGCC

TCGGCAAAAAGGCAGCCAGAATCGACGAACAGGAAAACTGGCAGGAAGGCAAAGAAAACATCAGAGCTGCTGTTG

CAGCAGGATGCAGGCAGATTCAAGACCTGGAGCTCTCTTCCGTGGAGGTGGATCCCTGTAGAGA**C**GCTCAGGCTG

CTGAGGAGGGCGCGGTGCTTGGTCTCTATGAATACGATGACCTAAAGCAAAAAAAGAAGATGGCTATGTCGG**C**GA

AGCTCTATGGAACTGGGGATCAGGAGGCCTGGCAGAAAGGAGTCCTGTTTGCTTCTGGGCAGAACTTGGCATGAT

GGAGACGCCAGCCAGCGAGATGATGC**A**AACCAGATTTGCCGAAATTATTGAGAAGAATCTCAAAAG**T**GCTAGTAG

TAAACCGAG**G**TTC**G**TATCAGACCCAGGTCTTGGATTGAGGAACAGGCAATGGGATCATTCCTCAGTGTGGCCAAA

GGATCTGA**T**GAGCCCTCAGTCTTCTTGGAAATTCACTACATAGGCAGCCCCAATGCAGACAAACCACCCCTTGTT

TGTTGGGAAAGGAATTACCTTTGACAGTGGTGGTATCTCCATCAAGGCTTCTGCAAATATGGACCTCATGAGGGC

TGACATGGGAGGAGCTACAACTATATGCTCAGCCATTGTGTCTGCTGCAAATCTCAGTTTGCCCATTAATATTAT

AGGTCTGGCCCCTCTGTGAG

Bp 327 of pTK-170/171:		C
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A
	Celera:	C
	Chimp sequence:	C
Bp 358 of pTK-170/171:		T
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	C
Bp 364 of pTK-170/171:		C
	NCBI-human 01.08:	G
	NCBI-human 02.07:	G
	Celera:	G
	Chimp sequence:	G
Bp 514 of pTK-170/171:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	T

	Celera:	T
	Chimp sequence:	C
Bp 597 of pTK-170/171:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	T
	Celera:	T
	Chimp sequence:	T
Bp 701 of pTK-170/171:		A
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	C
Bp 741 of pTK-170/171:		T
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	T
Bp 759 of pTK-170/171:		G
	NCBI-human 01.08:	T
	NCBI-human 02.07:	T
	Celera:	T
	Chimp sequence:	G
Bp 763 of pTK-170/171:		G
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A
	Celera:	A
	Chimp sequence:	A

Bp 833 of pTK-170/171: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

Bp 975 of pTK-170/171: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

pTK-172/173:

TAGGCAGCCCCAATGCAGACAAACCACCCCTTGTTTGTTGGGAAAGGAATTACCTTTGACAGTGGTGGTATCTCC

ATCAAGGCTTCTGCAAATATGGACCTCATGAGGGC**T**GACATGGGAGGAGCTACAACTATATGCTCAGCCATTGTG

TCTGCTGCAAATCTCAGTTTGCCCATTAATATTATAGGTCTGGCCCCTCTGTGAAAACATGCCCAGCGGCAAGGC

CAACAAGCTGGGGGATGTTGTTAGAGCCAGGAAC**G**GGAAGACCATCCAGGTTGGTAACACTGATGCTGAGGGGAG

GCTCATACTGGCTGATGCGCTCTGTTACGTGCACACATTTAACCCGAAGGTCATCCTCAATGCCACCACCTTAAC

AGGTGTCATAGATGTAGCTTTGGGGTCAGGTGCCACTGGGGTCTTTACCAATTCATCCTGGCTCTGGAACAAGCT

CTTCGAGGCCAGCATTGAAACAGGGGACCGTGTCTGGAGGATGCCTCTCTTCAAACATTGTACAAGACAGGTTGT

AGATTGCCAGCTGGCTGAT**A**TTAACAACATTGGAAAATATAGATCTGCGGGAGCATGTACATCTGCGGCATTCCT

GAAAGAATTCGTGACTCATCCTAAGTGGGCACATTTAGACATAGCAGGTGTGATGACCAACAAAGATGAGGTTCC

CTAT**T**TATGGAAAGGCATGACCGGGAGGCCCA**A**AAGGACTCTCATAGAGTTCTTACTTCGTTTCAGTCAAGACAA

TGCTTAGTTCAGATACTCAAAAATGTCTTCACTCT**A**TCTTAAATTGGACAGTTGAAGTTAAAAGGTTTTTGAATG

AATGGATGAAAATATTTTAAAGGAGGCAATTTATATTTAAAAATGTAGAACACAATGAAATTTTTATGCCTTGAT

TTTTTTTTCATTTTACACAAAGATTTATATGTTTTTTTTTTGAGATGGAGTCTC**G**CTCTGTCACCCAGGCTGGAG

TGCAG_TGGCATGATCTCAGCTCACTGCAACCTCCGCCTCCTAGGTTCAAGCGATTCTCCCACCTCAGCCACCTG

AATACCTGGGACTACAGG**C**GCCCACCACCATGCCCGGCTGATTTTTGTATTTTTAATGGAGACGGGGTTTCACCA

TATTGGCCAGGCTGGTCTCAAAACTCCTGACCCTGTGATCTGCCCGCCT**T**GGCCTCCCAAAGTGCTGGGATTACA

GGCGTAA**G**CCACCACGCCCGGCCAGTATATATTTTTAATTGAGAAGCAAAATTGTACTTCAGATTTGTGATGCTA

GGAACATGAGCAAACTGAAAATTACTAACCACTTGTCAGAAACAATAAATCCAACTTTTTGTGCAAAAAAAAAAA

AATACAAATATTAGCTGGGCATGGTGGTGCATGCCTGTAATCCCAGCTACTCGGGAGGCTGAGGCAGAATTGCTT

GAACCTGGGAGGCGGAGACTGCAGTGAGCTGAGATTGTGC**T**ACTGCTGACTTTGTCTCAAAAAACAAAACAAAAC

AAAAAAACAAAATGAAAACAAAAAGCCAGGGCTGCCTCTGCTCAATAATGTTCTATCTTTGTTCCGCCTCTTCTC

TGGGGTCTCACTTCTTGGGAGCG

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Bp 111 of pTK-172/173:                  T
                NCBI-human 01.08:       C
                NCBI-human 02.07:       C
                Celera:                 C
                Chimp sequence:         C

Bp 260 of pTK-172/173:                  G
                NCBI-human 01.08:       A
                NCBI-human 02.07:       A
                Celera:                 A
                Chimp sequence:         G

Bp 545 of pTK-172/173:                  A
                NCBI-human 01.08:       G
                NCBI-human 02.07:       G
                Celera:                 G
                Chimp sequence:         G

Bp 680 of pTK-172/173:                  T
                NCBI-human 01.08:       C
                NCBI-human 02.07:       C
                Celera:                 C
                Chimp sequence:         C

Bp 708 of pTK-172/173:                  A
                NCBI-human 01.08:       C
                NCBI-human 02.07:       C
                Celera:                 C
                Chimp sequence:         C

Bp 786 of pTK-172/173:                  A
                NCBI-human 01.08:       A
                NCBI-human 02.07:       G
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Celera: A

Chimp sequence: G

Bp 955 of pTK-172/173: G

NCBI-human 01.08: A

NCBI-human 02.07: A

Celera: A

Chimp sequence: G

Bp 981 of pTK-172/173: -

NCBI-human 01.08: G

NCBI-human 02.07: G

Celera: G

Chimp sequence: -

Bp 1069 of pTK-172/173: C

NCBI-human 01.08: T

NCBI-human 02.07: T

Celera: T

Chimp sequence: T

Bp 1175 of pTK-172/173: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

Bp 1208 of pTK-172/173: G

NCBI-human 01.08: G

NCBI-human 02.07: A

Celera: G

Chimp sequence: A

Bp 1351-1352 of pTK-172/173: AA

NCBI-human 01.08: --

NCBI-human 02.07: -

Celera: --

Chimp sequence: --

Bp 1466 of pTK-172/173: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

pTK-175/176:

CAGGGGACCGTGTCTGGAGGATGCCTCTCTTCAAACATTGTACAAGACAGGTTGTAGATTGCCAGCTGGCTGAT**A**
TTAACAACATTGGAAAATATAGATCTGCGGGAGCATGTACATCTG**C**GGCATTCCTGAAAGAATTCGTGACTCATC
CTAAGTGGGCACATTTAGACATAGCAGGTGTGATGACCAACAAAGATGAGGTTCCCTAT**T**TATGGAAAGGCATGA
CCGGGAGGCCCA**A**AAGGACTCTCATAGAGTTCTTACTTCGTTTCAGTCAAGACAATGCTTAGTTCAGATACTCAA
AAATGTCTTCACTCT**A**TCTTAAATTGGACAGTTGAAGTTAAAAGGTTTTTGAATGAATGGATGAAAATATTTTAA
AGGAGGCAATTTATATTTAAAAATGTAGAACACAATGAAATTTTTATGCCTTGATTTTTTTTTCATTTTACACAA
AGATTTATAT**G**TTTTTTTTTTT**T**GAGATGGAGTCTCGCTCTGTCACCCAGGCTGGAGTGCAG_TGGCATGATCTC
AGCTCACTGCAACCTCCGCCTCCTAGGTTCAAGCGATTCTCCCACCTCAGCCACCTGAATACCTGGGACTACAGG
CGCCCACCACCATGCCCGGCTGATTTTTGTATTTTTAATGGAGACGGGGTTTCACCATATTGGCCAGGCTGGTCT
CAAAACTCCTGACCCTGTGATCTGCCCGCCT**T**GGCCTCCCAAAGTGCTGGGATTACAGGCGTAA**G**CCACCACGCC
CGGCCAGTATATATTTTTAATTGAGAAGCAAAATTGTACTTCAGATTTGTGATGCTAGGAACATGAGCAAACTGA
AAATTACTAACCACTTGTCAGAA_CAATAAATCCAACTTTTTGTGCAAAAAAAAAAAA**AA**TACAAATATTAGCTG
GGCATGGTGGTGCATGCCTGTAATCCCAGCTACTCGGGAGGCTGAG_CAGAATTGCTTGAACCTGG_**A**_GCGGA
GACTGCAGTGAGCTGAGATTGTGC**T**ACTGCTGACTTTGTCTCAAAAA**CAAG**CAAACAAAAAACAAATGAAAACAA
AAAGCCAGGCTGCCTCTGCTCA**T**ATGTTCTATCTTTGTCCGCCTCTTCTCTGG**GTCTGCACTCTGGGAGCTGATG**
TGAGGTGAATTCTCTGAAGCTGACTGCCCTATCTGGACTCC

Bp 75 of pTK-175/176: A

NCBI-human 01.08: G

NCBI-human 02.07: G

Celera: G

Chimp sequence: G

Bp 210 of pTK-175/176: T

NCBI-human 01.08: C

NCBI-human 02.07: C

	Celera:	C
	Chimp sequence:	C
Bp 238 of pTK-175/176:		A
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	C
Bp 316 of pTK-175/176:		A
	NCBI-human 01.08:	A
	NCBI-human 02.07:	G
	Celera:	A
	Chimp sequence:	G
Bp 461 of pTK-175/176:		G
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A
	Celera:	A
	Chimp sequence:	G
Bp 473 of pTK-175/176:		T
	NCBI-human 01.08:	-
	NCBI-human 02.07:	-
	Celera:	-
	Chimp sequence:	-
Bp 513 of pTK-175/176:		G
	NCBI-human 01.08:	-
	NCBI-human 02.07:	G
	Celera:	-
	Chimp sequence:	-

Bp 601 of pTK-175/176:		C
	NCBI-human 01.08:	T
	NCBI-human 02.07:	T
	Celera:	T
	Chimp sequence:	T
Bp 707 of pTK-175/176:		T
	NCBI-human 01.08:	C
	NCBI-human 02.07:	C
	Celera:	C
	Chimp sequence:	C
Bp 740 of pTK-175/176:		G
	NCBI-human 01.08:	G
	NCBI-human 02.07:	A
	Celera:	G
	Chimp sequence:	A
Bp 849 of pTK-175/176:		-
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A
	Celera:	A
	Chimp sequence:	A
Bp 884-885 of pTK-175/176:		AA
	NCBI-human 01.08:	-
	NCBI-human 02.07:	-
	Celera:	-
	Chimp sequence:	-
Bp 849 of pTK-175/176:		-
	NCBI-human 01.08:	A
	NCBI-human 02.07:	A

Celera: A

Chimp sequence: A

Bp 947 of pTK-175/176: -

NCBI-human 01.08: G

NCBI-human 02.07: G

Celera: G

Chimp sequence: G

Bp 967-969 of pTK-175/176: -A-

NCBI-human 01.08: GAG

NCBI-human 02.07: GAG

Celera: GAG

Chimp sequence: GAG

Bp 999 of pTK-175/176: T

NCBI-human 01.08: C

NCBI-human 02.07: C

Celera: C

Chimp sequence: C

Bp 1103-1165 of pTK-175/176:

GTCTGCACTCTGGGAGCTGATGTGAGGTGAATTCTCTGAAGCTGACTGCCCTATCTGGACTCC

NCBI-human-01.08:
GGTCTCACTTCTTGGGAGCCTGTGTGAAGGTGAATTCCTCTGAAAGCTGACTGCCCCTATTTGGGACTCC

NCBI-human-02.07:
GGTCTCACTTCTTGGGAGCCTGTGTGAAGGTGAATTCCTCTGAAAGCTGACTGCCCCTATTTGGGACTCC

Celera:
GGTCTCACTTCTTGGGAGCCTGTGTGAAGGTGAATTCCTCTGAAAGCTGACTGCCCCTATTTGGGACTCC

Chimp::
GGTCTCACTTCTTGGGAGCCTGTGTGAAGGTGAATTCCTCTGAAAGCTGACTGCCCCTATTTGGGACTCC

Jagged1- reporter gene constructs:

pGL4.10-Jagged1-573:

AGGGGCGTGCCCAGGGTGAGCACGCCCTCTCATGAATATTAATAAGCGCGCATGCGCCCTGCCCGGCGTGCTGGG
TAGAGGTGGCCAGCCCCGGCCGCTGCTGCCAGACGGGCTCTCCGGGTCCTTCTCCGAGAGCCGGGCGGGCACGCG
TCATTGTGTTACCTGCGGCCGGCCCGCGAGCTAGGCTGGTTTTTTTTTTTCTCCCCTCCCTCCCCCCTTTTTCCA
TGCAGCTGATCTAAAAGGGAATAAAAGGCTGCGCATAATCATAATAATAAAAGAAGGGGAGCGCGAGAGAAGGAA
AGAAAGCCGGGAGGTGGAAGAGGAGGGGGAGCGTCTCAAAGAAGCGATCAGAATAATAAAAGGAGGCCGGGCTCT
TTGCCTTCTGGAACGGGCCGCTCTTGAAAGGGCTTTTGAAAAGTGGTGTTGTTTTCCAGTCGTGCATGCTCCAAT
CGGCGGAGTATATTAGAGCCGGGACGCGGCGGCCGCAGGGGCAGCGGCGACGGCAGCACCGGCGGCAGCACCAGC
GCGAACAGCAGCGGCGGCGTCCCGAGTGCCCGCGGCGCGCGGCGCAGCG

pGL4.10-Jagged1-1700:

CAAACCGGCCGCTGAATAGTCACGCTTTTCTGCAGGACATACCTACTATTAGGGCCAAAACTTTGTCCACCCTTC
AAAGGAAGTCGATGTTTCCATATAAAGGTCCCCTCAAATGCAACAGCAAGCCCGTGGGAGAGGGGTTAGCAGAGG
CTACGGCCAGCCCACGGGCTTTCTTCGACCTTGATTATGACCAGGAGTGTAGCTGTTAATTGCGAGGCTTGCCTC
AAGGTGGAAAACAGTATCGGTTTCCACTGCCACCCCAGAGAAGGCAAGTTCCGCGGCTGGCGGTGCTGGGGACAC
GGTCCCTCCCAGGCCCATCTCTTGCCACCCAGAGAGCTGCTCGGAGGCCGCCTACAGGTGCAATCCCGGCACTGC
GGCCGGGGCGTCGGGCCGGGGAGGGCGTCCAAGCCCACCAGCATCTCCGCCGGCCCTTCCCAAAGCCTGAACAGG
GCCCCGGCGTGCCCGCCGCCTTCTACCCCCGGTTTCCCCGCGCCTCTGCCCCGGCGCGGTTTGGATAGGAAGCTG
GGAGCCCTCCCAGGCTCCGCAGACTCGGATTTGGGAGGGGGTGGGACGCGGCCGAGGCTTCCCCTCGAATCTGCG
GCAAGCCTGGCTCCAGGAAAGTTTTTCAAAGTTCCCAGCAGCGTCTGCCCAGGTCGCCTCCGCGGGGCGAGCAGA
CGGCGGCAAGCGCGCCAGCCTCGCCGCCGCCTCTGCCGCCAGCAGAGCGCTCTGGGCGGCTCGCTCGCGGGAAGC
GGGCCGAACTCCCGGCGGGCAGGCAGGCCCTCCTCCCGGGGCGAAAGCCGCAGCTGACGCAGGCGGTTCGGAAGG
CGGAAGCTGCCCCGCTCCGACCGCTCAGTCAGCGCCGCGGCGCCTACACCTGGGGCCCCGACGCGCGGGCAAAGG
CGCACGGCCCGGGGCGCCCGAGGGGGCGGTCCCCGCTGGGGGCCTCCAGGCGTCCCTGAGCAACGATCCCTTCCA
AGTACCTCCCCGCACTCTCCCTTCCCTCCTGGCCCGAAGCTCCCGAGGGCGGGGGTTGGTGTGGGGCCCTGGTTC
TTCTACGCCGCCCTGAGCATCCCGCTGCCCCCAACCCCTTCCAAGTTCCTCCTCGCACTACCCCCTCCCCAGCAA
CGTGAAGGGGAGGGGCGTGCCCAGGGTGAGCACGCCCTCTCATGAATATTAATAAGCGCGCATGCGCCCTGCCCG
GCGTGCTGGGTAGAGGTGGCCAGCCCCGGCCGCTGCTGCCAGACGGGCTCTCCGGGTCCTTCTCCGAGAGCCGGG
CGGGCACGCGTCATTGTGTTACCTGCGGCCGGCCCGCGAGCTAGGCTGGTTTTTTTTTTTCTCCCCTCCCTCCCC
CCTTTTTCCATGCAGCTGATCTAAAAGGGAATAAAAGGCTGCGCATAATCATAATAATAAAAGAAGGGGAGCGCG
AGAGAAGGAAAGAAAGCCGGGAGGTGGAAGAGGAGGGGGAGCGTCTCAAAGAAGCGATCAGAATAATAAAAGGAG
GCCGGGCTCTTTGCCTTCTGGAACGGGCCGCTCTTGAAAGGGCTTTTGAAAAGTGGTGTTGTTTTCCAGTCGTGC
ATGCTCCAATCGGCGGAGTATATTAGAGCCGGGACGCGGCGGCCGCAGGGGCAGCGGCGACGGCAGCACCGGCGG
CAGCACCAGCGCGAACAGCAGCGGCGGCGTCCCGAGTGCCCGCGGCGCGCGGCGCAGCG

pGL4.10-TK-FL:

GGCCTAACTGGCCGGTACCTGAGCTCGCTAGCCTCGAGGATATCAAGATCTAAATGAGTCTTCGGACCTCGCGGG
GGCCGCTTAAGCGGTGGTTAGGGTTTGTCTGACGCGGGGGGAGGGGGAAGGAACGAAACACTCTCATTCGGAGGC
GGCTCGGGGTTTGGTCTTGGTGGCCACGGGCACGCAGAAGAGCGCCGCGATCCTCTTAAGCACCCCCCCGCCCTC
CGTGGAGGCGGGGGTTTGGTCGGCGGGTGGTAACTGGCGGGCCGCTGACTCGGGCGGGTCGCGCGCCCCAGAGTG
TGACCTTTTCGGTCTGCTCGCAGACCCCCGGGCGGCGCCGCCGCGGCGGCGACGGGCTCGCTGGGTCCTAGGCTC
CATGGGGACCGTATACGTGGACAGGCTCTGGAGCATCCGCACGACTGCGGTGATATTACCGGAGACCTTCTGCGG
GACGAGCCGGGTCACGCGGCTGACGCGGAGCGTCCGTTGGGCGACAAACACCAGGACGGGGCACAGGTACACTAT
CTTGTCACCCGGAGGCGCGAGGGACTGCAGGAGCTTCAGGGAGTGGCGCAGCTGCTTCATCCCCGTGGCCCGTTG
CTCGCGTTTGCTGGCGGTGTCCCCGGAAGAAATATATTTGCATGTCTTTAGTTCTATGATGACACAAACCCCGCC
CAGCGTCTTGTCATTGGCGAATTCGAACACGCAGATGCAGTCGGGGCGGCGCGGTCCCAGGTCCACTTCGCATAT
TAAGGTGACGCGTGTGGCCTCGAACACCGAGCGACCCTGCAGCGACCCGCTTAAAAGCTTGGCAATCCGGTACTG
TTGGTAAAGCCACCATGGAAGATGCCAAAAACATTAAGAAGGGCCCAGCGCCATTCTACCCACTCGAAGACGGGA
CCGCCGGCGAGCAGCTGCACAAAGCCATGAAGCGCTACGCCCTGGTGCCCGGCACCATCGCCTTTACCGACGCAC
ATATCGAGGTGGACATTACCTACGCCGAGTACTTCGAGATGAGCGTTCGGCTGGCAGAAGCTATGAAGCGCTATG
GGCTGAATACAAACCATCGGATCGTGGTGTGCAGCGAGAATAGCTTGCAGTTCTTCATGCCCGTGTTGGGTGCCC
TGTTCATCGGTGTGGCTGTGGCCCCAGCTAACGACATCTACAACGAGCGCGAGCTGCTGAACAGCATGGGCATCA
GCCAGCCCACCGTCGTATTCGTGAGCAAGAAAGGGCTGCAAAAGATCCTCAACGTGCAAAAGAAGCTACCGATCA
TACAAAAGATCATCATCATGGATAGCAAGACCGACTACCAGGGCTTCCAAAGCATGTACACCTTCGTGACTTCCC
ATTTGCCACCCGGCTTCAACGAGTACGACTTCGTGCCCGAGAGCTTCGACCGGGACAAAACCATCGCCCTGATCA
TGAACAGTAGTGGCAGTACCGGATTGCCCAAGGGCGTAGCCCTACCGCACCGCACCGCTTGTGTCCGATTCAGTC
ATGCCCGCGACCCCATCTTCGGCAACCAGATCATCCCCGACACCGCTATCCTCAGCGTGGTGCCATTTCACCACG
GCTTCGGCATGTTCACCACGCTGGGCTACTTGATCTGCGGCTTTCGGGTCGTGCTCATGTACCGCTTCGAGGAGG
AGCTATTCTTGCGCAGCTTGCAAGACTATAAGATTCAATCTGCCCTGCTGGTGCCCACACTATTTAGCTTCTTCG
CTAAGAGCACTCTCATCGACAAGTACGACCTAAGCAACTTGCACGAGATCGCCAGCGGCGGGGCGCCGCTCAGCA
AGGAGGTAGGTGAGGCCGTGGCCAAACGCTTCCACCTACCAGGCATCCGCCAGGGCTACGGCCTGACAGAAACAA
CCAGCGCCATTCTGATCACCCCCGAAGGGGACGACAAGCCTGGCGCAGTAGGCAAGGTGGTGCCCTTCTTCGAGG
CTAAGGTGGTGGACTTGGACACCGGTAAGACACTGGGTGTGAACCAGCGCGGCGAGCTGTGCGTCCGTGGCCCCA
TGATCATGAGCGGCTACGTTAACAACCCCGAGGCTACAAACGCTCTCATCGACAAGGACGGCTGGCTGCACAGCG
GCGACATCGCCTACTGGGACGAGGACGAGCACTTCTTCATCGTGGACCGGCTGAAGAGCCTGATCAAATACAAGG
GCTACCAGGTAGCCCCAGCCGAACTGGAGAGCATCCTGCTGCAACACCCCAACATCTTCGACGCCGGGGTCGCCG
GCCTGCCCGACGACGATGCCGGCGAGCTGCCCGCCGCAGTCGTCGTGCTGGAACACGGTAAAACCATGACCGAGA
AGGAGATCGTGGACTATGTGGCCAGCCAGGTTACAACCGCCAAGAAGCTGCGCGGTGGTGTTGTGTTCGTGGACG
AGGTGCCTAAAGGACTGACCGGCAAGTTGGACGCCCGCAAGATCCGCGAGATTCTCATTAAGGCCAAGAAGGGCG
GCAAGATCGCCGTGTAATAATTCTAGAGTCGGGGCGGCCGGCCGCTTCGAGCAGACATGATAAGATACATTGATG
AGTTTGGACAAACCACAACTAGAATGCAGTGAAAAAAATGCTTTATTTGTGAAATTTGTGATGCTATTGCTTTAT
TTGTAACCATTATAAGCTGCAATAAACAAGTTAACAACAACAATTGCATTCATTTTATGTTTCAGGTTCAGGGGG
AGGTGTGGGAGGTTTTTTAAAGCAAGTAAAACCTCTACAAATGTGGTAAAATCGATAAGGATCCGTCGACCGATG
CCCTTGAGAGCCTTCAACCCAGTCAGCTCCTTCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTTATGACT
GTCTTCTTTATCATGCAACTCGTAGGACAGGTGCCGGCAGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCTGCG
CTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGA

TAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTT
TTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGG
ACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGG
ATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGT
GTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAA
CTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAG
AGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGAACAGTATT
TGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCAC
CGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTT
GATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAA
AAGGATCTTCACCTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAACTTG
GTCTGACAGCGGCCGCAAATGCTAAACCACTGCAGTGGTTACCAGTGCTTGATCAGTGAGGCACCGATCTCAGCG
ATCTGCCTATTTCGTTCGTCCATAGTGGCCTGACTCCCCGTCGTGTAGATCACTACGATTCGTGAGGGCTTACCA
TCAGGCCCCAGCGCAGCAATGATGCCGCGAGAGCCGCGTTCACCGGCCCCCGATTTGTCAGCAATGAACCAGCCA
GCAGGGAGGGCCGAGCGAAGAAGTGGTCCTGCTACTTTGTCCGCCTCCATCCAGTCTATGAGCTGCTGTCGTGAT
GCTAGAGTAAGAAGTTCGCCAGTGAGTAGTTTCCGAAGAGTTGTGGCCATTGCTACTGGCATCGTGGTATCACGC
TCGTCGTTCGGTATGGCTTCGTTCAACTCTGGTTCCCAGCGGTCAAGCCGGGTCACATGATCACCCATATTATGA
AGAAATGCAGTCAGCTCCTTAGGGCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCGGTGTTGTCGCTCATGGTA
ATGGCAGCACTACACAATTCTCTTACCGTCATGCCATCCGTAAGATGCTTTTCCGTGACCGGCGAGTACTCAACC
AAGTCGTTTTGTGAGTAGTGTATACGGCGACCAAGCTGCTCTTGCCCGGCGTCTATACGGGACAACACCGCGCCA
CATAGCAGTACTTTGAAAGTGCTCATCATCGGGAATCGTTCTTCGGGGCGGAAAGACTCAAGGATCTTGCCGCTA
TTGAGATCCAGTTCGATATAGCCCACTCTTGCACCCAGTTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCG
GGGTGTGCAAAAACAGGCAAGCAAAATGCCGCAAAGAAGGGAATGAGTGCGACACGAAAATGTTGGATGCTCATA
CTCGTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTACTAGTACGTCTCTCAAGGATAAGTAAGTAATATT
AAGGTACGGGAGGTATTGGACAGGCCGCAATAAAATATCTTTATTTTCATTACATCTGTGTGTTGGTTTTTTGTG
TGAATCGATAGTACTAACATACGCTCTCCATCAAAACAAAACGAAACAAAACAAACTAGCAAAATAGGCTGTCCC
CAGTGCAAGTGCAGGTGCCAGAACATTTCTCT

Curriculum vitae

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