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DISSERTATION

High throughput technologies to investigate the molecular basis of congenital limb malformation

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Abstract

Genetische Veränderungen, wie Punkmutationen und Strukturvarianten, sind als Ursache für angeborene Defekte der Gliedmaßen im Menschen bekannt. Da es sich um ein phänotypisch und genotypisch sehr heterogenes Krankheitsbild handelt, ist es sinnvoll diese Fälle mit genomweiten Analyseverfahren wir der Microarray-basierten komparativen genomischen Hybridisierung (Array CGH) und mittels Exome Sequenzierung zu untersuchen.

Im ersten Teil dieser Arbeit habe, habe ich eine homozygote Missens-Mutation und eine überlappende homozygote Deletion in der Sterile Alpha Motif (SAM) Domäne des *ZAK* Gens identifiziert in einer pakistanischen und einer tunesischen Familie. Beide Familien zeigten Spaltfüße, Nagelverdopplung der Hände und Schwerhörigkeit. Wir konnten mittels in situ Hybridisierung zeigen, dass in der Maus ZAK im embryonalen Herzen und in den Extremitäten exprimieret ist. Der komplette knock out von beiden Isoformen von *ZAK* mittels CRISPR/Cas genome editing zeigte sich als früh embryonal letal. Die spezifische Deletion der SAM Domäne des *ZAK* Gens zeigte einen Duplikationsdefekt der Extremitäten, welcher mit einer verminderten *Tp63* Expression einherging.

Im zweiten Teil der Arbeit habe ich eine Familie aus dem Iran mit einer schweren Form der Synpolydaktylie mittels Exome Sequenzierung untersucht. Wir konnten erstmals eine homozygote Missens-Mutation im HOXD13 Gen als ursächlich für eine schwere Synpolydaktylie nachweisen. Mittels Electrophoretic Mobility Shift Assay (EMSA) konnten wir weiterhin zeigen, dass es sich um eine Loss of Function Mutation handelt.

Im dritten Teil dieser Arbeit habe ich 134 Familien mit Spalthänden und Spaltfüßen mittels Array CGH auf Deletionen und Duplikationen von Chromosom 7q21.3 untersucht. Dort liegt im *DYNC1I1* Gen ein bekannter kodierender Extremitäten Enhancer von *Dlx5/6*.

Zusammenfassend habe ich in dieser Arbeit folgende Ergebnisse erzielt:

1) Ich konnte Mutationen und Deletionen von *ZAK* als molekulare Ursache von Spalthänden identifizieren.

2) Ich konnte zeigen, dass eine homozygote Missens-Mutation im HOXD13 Gen als ursächlich für eine schwere Synpolydaktylie ist.

3) Ich konnte zeigen, dass Deletionen eines kodierenden Extremitäten Enhancers von *Dlx5/6 auf* Chromosom 7q21.3 in ca. 3% aller Familien mit Spalthänden und Spaltfüßen zu finden sind.

Abstract

Genomic alterations, such as point mutations and structural variations are known causes of congenital limb defects in human. The genetic heterogeneity underlying these anomalies requires a genome-wide diagnostic approach. In this thesis we applied the whole exome sequencing (WES) and microarray-based comparative genomic hybridization (array CGH) techniques in a group of patients presenting with different types of limb malformations.

The first part of the thesis concerns investigating a missense mutation and a deletion within the Sterile Alpha Motif (SAM) domain of the *ZAK* gene, that were identified in Pakistani and Tunisian families, respectively. All the affected family members in both pedigrees were presenting with split foot malformation, hearing impairment and nail deformity. We applied the CRISPR/Cas9 system in the mouse embryonic stem cells to generate a ZAK mouse model deficient in the SAM-containing domain of the gene. The obtained mutant showed a complex limb duplication defect. The abnormality was induced by the downregulation on the *Tp63* gene. Also, we showed that the *ZAK* gene was expressed in the heart and limbs in mice and the knockout of both ZAK isoforms via CRISPR/Cas9 genome editing was lethal in transgenic mice. The next section of the thesis concerns an Iranian patient presenting with a severe metacarpal-to-carpal transformation that was subjected to the whole exome sequencing. The patient's parents were consanguineous and not affected, which strongly indicated to a recessive mode of inheritance. WES analysis revealed a novel homozygous missense mutation c.938C>G (p.313T>R) in the *HOXD13* gene. We showed using electrophoretic mobility shift assay (EMSA) that this substitution led to the HOXD13 protein loss of function. The last chapter concerns a cohort of patients presenting with ectrodactyly. In this part, 134 families were subjected to array CGH. Heterozygous microdeletions encompassing two exons of the *DYNC1I1* gene, which normally functions as a limb enhancer of the *Dlx5/6* genes, on chromosome 7q21.3 were identified.

Based on the research described in this thesis we can conclude: 1) the crucial role of *ZAK* gene in limb deformities, 2) the causative character of the homozygous point mutation in the *HOXD13* gene identified in a patient presenting with a severe limb defect, whose healthy parents were carriers of the mutation, 3) the substantial role of deletions in coding extremities enhancer of *Dlx5/6* gene on chromosome 7q21.3, which were found in about 3% of all families with limb malformations.

Introduction

Ectrodactyly or split-hand/foot malformation (SHFM) affects the central rays of hands and/or feet. Approximately 1 in 18,000 infants are born with these defects. Genetic alterations affecting the *Tp63,* the *Dlx5/6* and the *WNT10B* genes were up till now identified as the underlying cause in a large proportion of the affected individuals. Studying of the aforementioned genes gave an insight into their role in the limb development. Nevertheless, many patients presenting with limb defects remain without a molecular diagnosis (Czeizel et al., 1993).

The zipper sterile-alpha-motif kinase (*ZAK*) gene belongs to a mixed lineage protein kinase (MLK) family, which shares several distinctive structural features. There are two splicing variants, *ZAK-α* and *ZAK-β*. Both variants share the kinase catalytic domain, that contain motifs conserved in the serine/threonine and tyrosine kinase proteins, as well as a single leucine/isoleucine zipper motif containing domain. *ZAK-α* has a longer C-terminal region containing a Sterile-α motif (SAM) domain, which is absent in the *ZAK-β* variant. Gotoh et al. (2001) indicated that SAM domain has a role in protein-protein interaction in the mammalian cells. So far, there was no convincing evidence proving the role of the *ZAK* gene in the limb bud development.

Synpolydactyly (SPD1) (OMIM 186000) is a limb abnormality manifested by a postaxial polydactyly and a cutaneous webbing between the third and fourth digits. SPD1 arises from the mutations in the *HOXD13* gene and is inherited as an autosomal dominant trait. The causative mutations can be categorized in the three groups: loss-of-function (LOF) mutations (e.g. nonsense or frameshift), expansions of the N-terminal polyalanine repeat, and missense mutations. In our research, for the first time, a homozygous point mutation in the *HOXD13* gene was reported in the patient presenting with the severe metacarpal-to-carpal transformation. Noteworthy, there was no noticeabe phenotype in the parents heterozygous for the mutation.

There are studies showing that some coding sequences can function as enhancers regulating their neighboring genes rather than the genes they reside in. Such coding sequences are called exonic enhancers (eExons) (Ritter et al., 2012). One example is the limb enhancers driving the expression of the *Dlx5* and *Dlx6* genes, that are a part of the coding sequence of the *DYNC1I1* gene. Interaction of those regions with the *Dlx5/6* promoters were observed both in zebrafish and mice. However, the two exons of the *DYNC1I1* gene (exons 15 and 17) have an essential role in the brain development and there is no proof of their involvement in the limb bud development (Birnbaum et al., 2012a; Birnbaum et al., 2012b).

Objective

The general aim of this thesis is to identify genes or regulatory elements (REs) that play an important role in the limb malformation.

The specific aims are:

1. To identify mutations in the *ZAK* gene as the underlying cause of the split feet-like malformations, nail abnormality of hands and hearing loss in two consanguineous families (Pakistani and Tunisian) and to further investigate the role of *ZAK* gene in limb development.
2. To detect a homozygous point mutation in the *HOXD13* gene as the underlying cause of severe synpolydactyly.
3. To investigate deletions of two exons (15 and 17) of the *DYNC1I1* gene, which function as exonic enhancers, in 134 families presenting with ectrodactyly.

Methods

Whole exome Sequencing (WES)

Genomic DNA was isolated from the peripheral blood samples, and following Agilent SureSelect All Exon kit V4 enrichment the samples were subjected to the Next Generation Sequencing (paired end 150bp). Next, the Phenotypic Interpretation of eXomes (PhenIX) software was utilized to prioritize the candidate genes and to evaluate variants based on the population frequency, and the predicted pathogenicity. A ranking of the genes was created according to a variant score and a clinical relevance score (Zemojtel et al., 2014) .

Sanger sequencing

PCR was performed in a total volume of 20 µl with 40 ng genomic DNA as a template, 2 µl 10x PCR buffer, 0.6 µl dNTP mix (10 mM), 0.5 µl primer (10 pMol/µl), 0.6 µl MgCl2 (50 mM) and 0.2 µl Taq polymerase (Rapidozym, Germany). The PCR products were purified by enzymatic treatment (Exonuclease I, NEB; Shrimp Alkaline Phosphatase, Roche Diagnostics). BigDye v3.1 (Applied Biosystems) sequencing kit was used for sequencing of the PCR products. PCR products were then analysed by capillary automat ABI3730 (Applied Biosystems). The sequencing results were processed by DNA-STAR software (DNA-Star).

Microarray-based comparative genomic hybridization (array CGH)

Genomic DNA was extracted from blood samples. Array CGH was carried out using a whole genome 1M oligonucleotide arrays (Agilent, Santa Clara, CA). 1M arrays were analysed by Feature Extraction v9.5.3.1 and CGH Analytics v3.4.40 software or Cytogenomics v2.5.8.11 respectively (Agilent, Santa Clara, CA). Analysis settings: aberration algorithm: ADM-2; threshold: 6.0; window size: 0.2 Mb; filter: 5 probes, log2ratio = 0.29. The genomic profile was visualized by the Signal Map software (Signal Map v1.9.0.03, Nimble Gen Systems Inc.). Data were submitted to the DECIPHER database (http://decipher.sanger.ac.uk); accession numbers: BER284939, BER284938, BER284937 and BER285016.

Quantitative real-time PCR (qPCR) for copy number variations (CNVs)

QPCR was carried out as described previously (Klopocki et al., 2012) using genomic DNA of the affected and unaffected members to confirm the structural abnormalities in the families. The primers for the region of interest and two control primer pairs (human/mouse Albumin and F8) were designed.

For each primer pair, the reaction was done in triplicates for all samples. The primers were diluted to 100pmol/µl; each pair (i.e. forward and reverse) was then mixed together in a 1:1 ratio and diluted 1:40 in ddH2O. For individual reactions, 10ng of genomic DNA was required. The master mix for one sample included: 6µl Sybr green, 1µl Primer mix and 10µl ddH2O. The DNA and master mix were pipetted on a 384 well plate, centrifuged for 30sec at 1200rpm and placed in the RT qPCR cycler.

Quantitative PCR (qPCR) for mRNA expression

The lysis of samples was performed with the use of Trizol (Invitrogen) and phenol and chloroform were utilized for RNA extraction. cDNA was obtained in the reverse transcription reaction by means of the cDNA Synthesis Kit (Taqman Gold). SYBR Green (Applied Biosystems) was used to detect gene expression profiles in different human and mouse samples. The evaluation was performed by relative quantification, i.e related to a calibrator in an ABI Prism 7500 thermal cycler (Applied Biosystems Foster City, US).

Electrophoretic Mobility Shift Assays (EMSA)

Cloning and expression of wildtype and mutant homeodomains in *E.coli* were performed as described previously (Ibrahim et al., 2013). EMSAs were performed using 20 µl binding buffer (100 mM NaCl, 2 mM MgCl2, 0.1 mg/mL BSA, 4 mM spermidine, 25 mM HEPES, pH 7.5, Roche complete protease inhibitor), and 1.5 µL poly (dIdC) (100 ng/µL) as well with varying amounts of purified homeodomain (3, 6, 9, 12, 15 ng) were added. Reactions were incubated on ice for 5 min, then 1µl of the Cy3-labeled double-stranded oligonucleotide (5'CY3-ggatcCCAATAAAAtcggc-3') (0.75 pmol/µl) followed by 15 min incubation at room temperature (20°C). Before loading of the samples onto the gel, 2µl loading buffer (40% glycerol + 0.01% bromophenol blue) were added. Electrophoresis was performed on an 8% native polyacrylamide gel in 1x TBE and scanned on a FLA-5000 Scanner (Fuji).

Whole Mount In Situ Hybridization (WISH)

WISH for *ZAK*, *Fgf8*, *Tp63* and *Shh* was carried out on the wild-type (WT) embryos (C57/Bl6J) and mutant embryos at embryonic stages E10.5. WISH was carried out as described previously (Schwabe et al., 2004; Stricker et al., 2006).

Skeletal staining

Skeletal staining of the E18.5 WT and mutant embryos was performed using alcian blue and alizarin red as previously described (Mundlos, 2000).

CRISPR sgRNA selection and cloning

The designed sgRNAs were flanking the targeted region. We used the http://crispr.mit.edu/ platform to obtain candidate sgRNA sequences with low off-target scores. Complementary strands were annealed, phosphorylated, and cloned into the BbSI site of pX459 or pX330 CRISPR/Cas9 vector. One experimental design led to a complete inactivation of the *ZAK* gene through interrupting the sequence of the 2nd exon of the gene. The other experimental design led to a deletion of the SAM domain of *ZAK* (exons 12-16) with the use of two sgRNAs that introduced lesions upstream and downstream of the target region.

Mouse embryonic stem cell (mESC) culture and transfection

3×10^5 G4 mESC were seeded on the plates with the CD1 feeder cells and transfected with 8 µg of each CRISPR/Cas9 construct using FuGENE technology (Promega). When the pX330 construct was used the cells were co-transfected with a puromycine resistant plasmid. PX459 in contrast already contains a puromycine resistant cassette, no additional puromycine resistant plasmid was needed. 24 hours after transfection, cells were split and transferred into plates with the DR4 puro-resistant feeders and selected with puromycine for the next 48h. Clones were then grown for 5 to 6 more days, picked, and transferred into 96-well plates with the CD1 feeder cells. After 2 days of culture, the cells were split in triplicates, two parts were frozen and one part was kept for growth and DNA harvesting. After selecting positive clones, plates containing the CD1 feeder cells were prepared. Positive clones were thawed and grown on the CD1-feeder plates until they reached an average density of four million cells. Two to three vials of cells were frozen and stored in the liquid nitrogen. DNA was harvested from the rest of the cells and genotyping for confirmation was performed.

PCR-Based Genotyping

Primers for the allele specific PCR were designed in the distance of 100-300 bp flanking the cutting sites of the sgRNAs' target regions. Standard PCR and electrophoresis steps were performed on the high number of DNA samples form the screened clones. Clone selection was based on the presence of the allele specific product on the agarose gel. In the CRISPR/Cas9 experiment aiming at the complete *ZAK* inactivation, selected PCR products were later Sanger-sequenced. In the experiment aiming at the SAM domain exclusion qPCR for the copy number variations in the target region was performed.

Mouse aggregation

A frozen vial of mESCs was thawed, cells were seeded on a plate containing the CD1 feeders and grown for 2 days. Mice were generated by the diploid or tetraploid aggregation of positive clones as described before (Artus and Hadjantonakis, 2011). All animal procedures were in accordance with institutional, state, and government regulations (Berlin: LAGeSo).

Results

In the first part of my thesis, we studied an autosomal-recessive syndrome with split feet-like phenotype, nail abnormalities in the hands and hearing impairment. To identify the genetic cause of this disorder, genome-wide linkage analysis followed by the whole exome sequencing (WES) were carried out in a consanguineous Pakistani family with four individuals affected. Autozygosity mapping following a single nucleotide polymorphism (SNP) array-based genotyping of five individuals identified a disease-associated locus on chromosome 2q31, with a maximum LOD score of 3.5. After performing the WES in one of the affected individuals (VI: 3) and filtering for rare and potentially damaging variants, two homozygous variants were detected in the 9.1 Mb linkage region. The first variant was c.1247T>A (p.Phe416Tyr) in the *ZNF385B* gene and the second variant c.1103T>G (p.Phe368Cys) in the *ZAK* gene. Both variants were predicted to be damaging, they were absent or very rare in the Pakistani control cohort and the EXaC browser, and none of the genes had a known function in the limb development. In order to prioritize these candidate genes for further studies, we performed the whole-mount in situ hybridization on mouse embryos using probes specific to the *Znf385b* and *ZAK* genes. While the *Znf385b* gene was mainly expressed in the developing brain at embryonic day E10.5 with limited expression in limbs, the *ZAK* gene was consistently expressed in the heart and the developing forelimb and hindlimb between embryonic days E9.5 and E11.5, making it a plausible candidate gene for the studied limb phenotype. To obtain additional evidence for the involvement of the *ZAK* gene mutations in SHFM in humans, we sequenced its coding sequence and the splice sites in 106 unrelated individuals presenting with the wide clinical SHFM spectrum. In the result we were able to detect genetic alteration in the *ZAK* gene in a second SHFM patient, a Tunisian boy. Using high-resolution array CGH, we identified a homozygous intragenic deletion of part of the *ZAK* gene, which was heterozygous in his first cousin parents. The 14.7 kb deletion removing exons 12-16 of the *ZAK* gene was confirmed with qPCR and the exact breakpoints were identified by Sanger-sequencing. No comparable deletion was present in over 600 individuals included in our in-house database of the copy number variants as well as the DECIPHER database (http://decipher.sanger.ac.uk) (Firth et al., 2009). Noteworthy, the missense mutation and the intragenic deletion both affected the SAM domain of the *ZAK* gene are only presented in ZAK-α variant. To elucidate the molecular function of the *ZAK* gene and establish its possible involvement in the limb development, we decided to utilize the CRISPR/Cas9 genome editing machinery by introducing mutation in the mouse *ZAK* gene. First, we planned to create a complete knockout of both

isoforms of *ZAK* in mice. Therefore, we designed one single guide RNA (sgRNA) specific to the second exon of the *ZAK* gene. After transfecting the mouse embryonic stem cells (ESCs) with the previously prepared CRISPR/Cas9 construct, we screened 96 clones for mutations via Sanger sequencing. Approximately 60% of the clones carried a homozygous frameshift mutation, 20% carried a heterozygous mutation and 20% were wild type. For further studies, we selected two clones with homozygous frameshift mutations occurring in exon 2, disrupting both isoforms of *ZAK***.** Next, we performed diploid ES cells aggregation of the two clones to produce highly chimeric animals. The CRISPR/Cas9 mediated complete inactivation of the *ZAK* gene in mice resulted in a full penetrant lethality at the embryonic day E9.5 due to a cardiac malformation**.** The homozygous mice showed a severe cardiac edema and a global growth retardation and were subsequently absorbed between embryonic days E9.5 and E10.5. No viable homozygous mice could be obtained. In contrast, the heterozygous mice were morphologically indistinguishable from their wild-type littermates. Since both of the identified genetic alterations were located in the sequence coding for the SAM domain, we assumed that the mutations restricted only to altering this functional domain in the ZAK-α isoform could be responsible for the observed limb malformations. Therefore, we aimed at genocoping the intragenic deletion identified in the Tunisian family and specifically delete the SAM domain of ZAK-α isoform using an adapted CRISPR/Cas9 protocol for the introduction of structural variants by inducing double strand breaks (DSBs) (Kraft et al., 2015). We designed two sgRNAs located in the introns centromeric and telomeric to exons 12 and 16 of the *ZAK* gene, respectively. After co-transfecting the mouse ESCs with two plasmids containing sgRNAs and a selection process, we screened 288 clones for deletions using a junction-fragment PCR-based approach and qPCR. We detected 3 clones with heterozygous and two clones with homozygous deletions of the 12 kb fragment of the target region. No predicted off-target mutations were found via Sanger sequencing. In chimeric animals generated from the selected two homozygous clones, we observed a spectrum of unilateral complex hindlimb duplication phenotypes with a low penetrance (4 out of 64 homozygous animals). At embryonic day E18.5, one supernumerary hindlimb with normal polarity was connected to an imperfectly duplicated pelvic girdle. The duplicated hindlimb showed shortened femur, fibular, tibia and a normal-sized foot. The forelimbs were normal. At later stages, variable splitting of the distal skeleton of the hindlimbs was observed**.** A five-week-old mouse showed a partial duplication of the right hindlimb and pelvic structures. While the foot was completely duplicated, the fibular and tibia were only partially duplicated and the femur shaft was split and only the distal part was duplicated.

Some parts of the pelvic girdle were also duplicated. Two mice showed polydactyly of the feet representing a minimal version of a distal duplication defect. To identify possible regulators or interaction partners of *ZAK*, a whole mount *in situ* hybridization (WISH) and an expression analysis were performed for some key developmental genes of early limb and digit development. WISH staining profile in the chimeric and homozygous embryos for *Fgf8, Shh* and *Tp63* were normal at the embryonic day E10.5. Taking into account previously published data regarding the possible correlation between *ZAK* and *Tp63* (Yang et al., 2006), we also performed a RT-qPCR for *Tp63* in mutant limb tissues. Expression analysis of the homozygous mutant hindlimbs at the developmental stages E10.5 and E11.5 showed indeed a 60% decrease in the *Tp63* expression compared to the wild type hindlimbs, suggesting a correlation between *ZAK* and *Tp63* expression in the developing limb.

In the second part of this research, the whole exome sequencing was performed on the Iranian patient presenting with the severe phenotype of synpolydactyly. Via PheniX analysis we detected a homozygous point mutation (c.938C>G) in the *HOXD13* gene located on chromosome 2. This mutation results in a threonine to arginine substitution at the position 313 of the HOXD13 protein. Since neither of the parents, who were first cousins, were affected we performed a co-segregation analysis using Sanger sequencing and confirmed both parents to be heterozygous and the index patient to be homozygous for the point mutation. We also performed EMSA with purified wildtype and T313R mutant homeodomains to investigate whether the mutated protein lost its ability to bind DNA. The results showed that *HOXD13*^{wt} bound the probe producing a sharp, shifted band on the gel. On the other hand, the *HOXD13*^{T313R} mutation did not produce a gel shift, and it indicated that the *HOXD13*^{T13R} homeodomain is not able to bind via *HOXD13* binding site.

Finally, a group of patients presenting with spectrum of the split hand/foot malformations were subjected to the array CGH testing. In four families, two German and two Polish, partially overlapping deletions were identified and all including the cis-regulatory *DYNC1I1* eExons that drive the *Dlx5/6* genes expression. Heterozygous deletions of the sizes of 167kb, 205kb, 169kb and 510kb were observed in these four families. In the family with a 510 kb deletion, apart from the SHFM, the affected individuals also suffered from a hearing impairment and the deleted region included not only the two exons of *DYNC1I1* gene that serve as the limb enhancers, but also a human brain enhancer called "hs1642", the entire sequence of the *C7orf76* gene and the last three exons of the *SLC25A13* gene.

Discussion

The correlation between mutations in *ZAK* gene and limb defects

ZAK (MLTK) gene belongs to the MAP triple kinase family, which has an important role in the gene expression, control of the cell growth and regulation of the cytoskeletal changes (Cheng et al., 2009; Yang, 2003). MAPKKK family members are serine/threonine protein kinases, which can activate and phosphorylate a dual specificity kinase, MAPKK, which in turn transfers phosphates onto threonine and tyrosine residues of a third enzyme, MAP kinase. The MAP kinase subsequently phosphorylates and activates various transcription factors. Although no role has yet been assigned to *ZAK* during limb development in vertebrates, the knockdown of the *ZAK* gene caused an extreme damage of craniofacial cartilage in *Xenopus lavis* (Gotoh et al., 2001; Suzuki et al., 2012). *ZAK* encodes two isoforms, the 800 amino acid-isoform alpha and the 455 amino acid-isoform beta. Both isoforms contain an N-terminal protein kinase domain followed by a leucine zipper. *ZAK* was shown to be a direct target of *TP63* binding and therefore a functional link was suggested (Yang et al., 2006).

CRISPR/Cas9 system, a recently developed genome editing technology created a new potential to successfully and time efficiently reengineer variable genomes. This allows for reconstructing specific human mutations and structural variations in animal models (Wang et al., 2013). We aimed at studying the effect of mutations in the *ZAK* gene in transgenic mice using the CRISPR/Cas9 genome editing technology. We used CRISPR/Cas9 system to create a mouse mutant deficient in the 12kb fragment of the *ZAK* gene including the region between exons 12 and 16. This was done by targeting two different genomic positions and inducing a deletion with the use of the CRISPR/Cas9 system in the mouse ESCs. By diploid or tetraploid aggregations of the selected clones positive for the homozygous deletion in the target region, chimeric animals were generated. The mice showed a range of a limb abnormal phenotypes, from a duplication of the left hindlimbs to polydactyly.

One of the biggest technical problems concerning the CRISPR/Cas9 system are the off-target mutations, i.e. mutations at undesired sites. Overall, there are multiple highly similar stretches of DNA in the genome which unpredicted cutting site can be created wrongfully through identification target region by the sgRNA. Therefore, for designing a CRSPR/Cas9 experiment it is crucial to choose the target sites with the lowest off-target score and to ensure the highest possible specificity of the guide RNA (Xiao et al., 2014). In our experimental design, we predicted two off-targets in the genes, which had not been linked to the limb development. Hence, we believe that the phenotype observed in the

mice carrying a homozygous deletion in the *ZAK* gene is strongly related to the created deletion and the disruption of this gene.

A literature review revealed that our *ZAK* mutant mice with the hindlimb duplication of one side looked highly similar to the mice treated embryonically with retinoic acid, which caused lower limb duplications and in some cases also hindlimb oligodactyly (Niederreither et al., 1996). Interestingly, it was shown that the treatment of limb bud cells with retinoic acid resulted in a dose dependent downregulation of *Tp63 in vitro* (Wang et al., 2014). In our expression analysis of the homozygous mutant hindlimbs at the embryonic days E10.5 and E11.5, a 60% decrease of the *Tp63* gene expression was observed comparing to the wild type hindlimbs. This suggests a correlation between *ZAK* and *Tp63* expression in the developing limb.

According to our results, we might suggest the role of *ZAK* alpha isoform in the development of the limb bud. Moreover, the deletion of the SAM domain in the isoform is linked to the downregulation of *Tp63* in the limb defects. But how does *Tp63* expression relate to the phenotypic differences between the human condition and the mutant mice? Mutations affecting the *Tp63* expression are connected to a wide range of limb phenotypes, the knockout of *Tp63* results in a severe truncation of both limbs while the knock-in (by homologous recombination of an ectrodactyly mutation R279H) leads to a complete reduction of the hindlimbs and milder reduction of the forelimbs (Vanbokhoven et al., 2011; Yang et al., 1999). Interestingly also some of the heterozygous mice showed a reduction phenotype indicating a dosage effect. In contrast, treatment of the mice with retinoic acid was shown to cause lower limb duplications, polydactyly, and also oligodactyly, at least partly due to the downregulation of *Tp63* (Niederreither et al., 1996; Wang et al., 2014). Therefore, we propose that mutations in *ZAK* cause a downregulation of *Tp63* by 60% in the hindlimbs giving rise to a wide spectrum of lower limb phenotypes: in humans they are associated with split feet malformation and in transgenic mice with lower limb duplications. We suggest the name "Osteodiakladosis" (ancient Greek: branched bones) for this phenotypic spectrum. It is also possible that *ZAK* influences the retinoic acid pathway directly and thereby contributes to the phenotype.

Altogether, we conclude that *ZAK* gene might play a role in limb modelling, and the mutations affecting the sequence coding for the SAM domain of the *ZAK* gene could lead to a recessive syndrome with the phenotypes of variable foot malformations, nail abnormalities and hearing impairment.

Loss of function of homozygous *HOXD13* mutation in the patient with the metacarpal-to-carpal transformation phenotype

Synpolydactyly (SPD) is a genetically heterogenous limb malformation. This defect presented in the wide range of limb phenotypes can be caused by heterozygous mutations in the *HOXD13* gene (Brison et al., 2014). To date, four families with individuals carrying homozygous *HOXD13* mutations have been reported. Heterozygous individuals usually show a SPD-phenotype with incomplete penetrance, while homozygous individuals are presented with an additional metacarpal-to-carpal transformation feature that is fully penetrant (Johnson et al., 2003). In our case of a severe SPD with metacarpal-to-carpal transformation, a homozygous missense mutation was detected in the *HOXD13* gene, coding for a DNA-binding domain.

The c.938C>G transition in exon 2 of *HOXD13* results in a p.T313R substitution, located at position 38 of the DNA-binding homeodomain of HOXD13. The replacement of threonine to arginine, a large and positively charged amino acid, probably causes an incorrect folding of the homeodomain and thus leads to a loss of function (LOF) effect on the mutant protein, as it was shown by our EMSA experiment.

Up till now, seven point mutations in the homeodomain of *HOXD13* gene have been considered as the pathogenic causes of limb malformation phenotypes ranging from SPD to brachydactyly. Three out of seven mutations caused LOF with incomplete penetrance (Dai et al., 2014; Debeer et al., 2002). While, the other four missense mutations are gain of function (GOF) alleles with brachydactyly type E, D and syndactyly type V phenotypes (Caronia et al., 2003; Johnson et al., 2003; Zhao et al., 2007). With respect to the dominant inheritance of all *HOXD13* mutations, our missense mutation, T313R is expected to have an autosomal-dominant effect with reduced penetrance, despite having no report of an affected family members heterozygous for the substitution.

In our research, a homozygous missense mutation in the *HOXD13* gene was described in a correlation with metacarpal-to-carpal transformation phenotype. So far, it is uncertain if the damaging effect of homozygous mutations in *HOXD13* results to a complete loss of protein function. Different research regarding mouse model presented inconsistent results for this scientific question. For instance, a homozygous *HOXD13* knockout mouse model revealed a reduced digit length without metacarpal-to-carpal transformation phenotype (Dolle et al., 1993). In contrast, the polyalanine expansions in the *HOXD13* gene in another animal model presented the metacarpal-to-carpal transformation, resembling the patient's phenotype (Johnson et al., 1998). The difference between mouse models and

human patients could be explained by the type of the introduced mutation. The *HOXD13* knockout mouse model does not produce any HOXD13 protein, not even a truncated one. On the other hand, the expansions of polyalanine, the human p.Q243X truncation and the p.T313R mutations in our patient most likely lead to the expression of a truncated HOXD13 protein that could contribute to create limb malformations by gaining new interactions.

The association between the deletions in eExons of *DYNC1I1* gene and limb malformations

Copy number variation analyses were performed in 134 families with SHFM showed that in 13% of cases were detected a duplication of the chromosomal region 17p13.3, in 12% of cases the 10q24 region was duplicated, in 4% of cases there were *TP63* mutations identified, and in 3% of cases were observed a deletion of two exons of the *DYNC1I1* gene (eExons). There was no known molecular diagnosis in 68% of cases in this SHFM cohort. Here, in four families presenting with limb malformations, we identified overlapping microdeletions in the chromosome 7q21.3 of various sizes, from 167 kb, 169 kb, 205 kb to 510 kb. The deletion included exons 15 and 17 of *DYNC1I1* gene, few exons of the *SLC25A13* gene and an eDlx#23 enhancer element. All four families shared a deletion of the two *DYNC1I1* gene exons. However, in the family with 510 kb deletion the affected members suffered from hearing loss, apart from the SHFM and their deletion additionally encompassed some exons of the *SLC25A13* gene, as well as the *C7orf76* gene, two branchial arch enhancers and the hs1642 enhancer. We observed the eDlx#23 element to be deleted in all four families. eDlx#23 was shown to lead to a reporter expression in forebrain, optic vesicle, limb bud mesenchyme and branchial arch (Birnbaum et al., 2012b), however only one out of four families revealed hearing loss phenotype.

In 2014, a deletion of the size of 106 kb including the *DYNC1I1* eExons and the eDlx#23 were reported in a family with the phenotype of SHFM without hearing loss (Lango Allen et al., 2014). According to this data, the lack of the eDlx#23 enhancer element is probably not the cause of hearing impairment in our case. Instead, we propose that the pathogenic cause of hearing loss in our family is likely due to the deleted 264 kb telomeric region involving hs1642, two branchial arch enhancers and *C7orf76* gene.

Taken together, one of the pathogenic causes of SHFM can be deletions of eExons 15 and 17 of the *DYNC1I1* gene, which have the regulatory role for *Dlx5* and *Dlx6* limb genes. However, the deletion of the hs1642 in the telomeric region is likely to be linked to the hearing impairment.

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Affidavit

I, Naeimeh Tayebi certify under penalty of perjury by my own signature that I have submitted the thesis on the topic “High throughput technologies to investigate the molecular basis of congenital limb malformation” I wrote this thesis independently and without assistance from third parties, I used no other aids than the listed sources and resources.

All points based literally or in spirit on publications or presentations of other authors are identified as such in the correct citation made. The sections on methodology (in particular practical work, laboratory requirements, statistical processing) and results (in particular images, graphics and tables) correspond to the URM (s.o) and are answered by me. My contributions in the selected publications for this dissertation correspond to those that are specified in the following joint declaration with the responsible person and supervisor. All publications resulting from this thesis and which I am author of correspond to the URM (see above) and I am solely responsible.

The importance of this affidavit and the criminal consequences of a false affidavit (section 156,161 of the Criminal Code) are known to me and I understand the rights and responsibilities stated therein.

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Date Signature

Naeimeh Tayebi had the following share in the following publications:

Publication 1:

Spielmann M, Kakar N, **Tayebi N**, Leettola C, Nürnberg G, Sowada N, Lupiáñez DG, Harabula I, Flöttmann R, Horn D, Chan WL, Wittler L, Yilmaz R, Altmüller J, Thiele H, van Bokhoven H, Schwartz CE, Nürnberg P, Bowie JU, Ahmad J, Kubisch C, Mundlos S, Borck G.
Exome sequencing and CRISPR/Cas genome editing identify mutations of ZAK as a cause of limb defects in humans and mice. Genome Res. 2016; 26(2):183-91 (Impact Factor: 14.6)

Contribution: N. Tayebi was involved in designing the study. Finding the second family with *ZAK* gene mutation and creating of mouse model were carried out by N.Tayebi. N.Tayebi also performed the data analysis and its interpretation and contributed in writing the manuscript as well.

Publication 2:

Ibrahim DM, **Tayebi N**, Knaus A, Stiege AC, Sahebzamani A, Hecht J, Mundlos S, Spielmann M.

A homozygous HOXD13 missense mutation causes a severe form of synpolydactyly with metacarpal to carpal transformation. Am J Med Genet A. 2016;170(3):615-21(Impact Factor: 2.15)

Contribution: N. Tayebi was involved in patient recruitment and phenotyping, NGS experiment and data analysis and also in writing and reviewing the manuscript.

Publication 3:

Tayebi N, Jamsheer A, Flöttmann R, Sowinska-Seidler A, Doelken SC, Oehl-Jaschkowitz B, Hülsemann W, Habenicht R, Klopocki E, Mundlos S, Spielmann M.

Deletions of exons with regulatory activity at the DYNC1I1 locus are associated with split-hand/split-foot malformation: array CGH screening of 134 unrelated families. Orphanet J Rare Dis. 2014; 9:108 (Impact Factor: 3.358)

Contribution: N. Tayebi was involved in array CGH experiments and analysis. N. Tayebi also contributed in writing and reviewing the manuscript.

Signature of the doctoral candidate _____

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Curriculum vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

Publications

[1] Spielmann M, Kakar N, **Tayebi N**, Leettola C, Nürnberg G, Sowada N, Lupiáñez DG, Harabula I, Flöttmann R, Horn D, Chan WL, Wittler L, Yilmaz R, Altmüller J, Thiele H, van Bokhoven H, Schwartz CE, Nürnberg P, Bowie JU, Ahmad J, Kubisch C, Mundlos S, Borck G**. Exome sequencing and CRISPR/Cas genome editing identify mutations of ZAK as a cause of limb defects in humans and mice.** Genome Res. 2016 Feb; 26(2):183-91. (IF: 14.6)

[2] Ibrahim DM, **Tayebi N**, Knaus A, Stiege AC, Sahebzamani A, Hecht J, Mundlos S, Spielmann M. **A homozygous HOXD13 missense mutation causes a severe form of synpolydactyly with metacarpal to carpal transformation.** Am J Med Genet A. 2016 Mar; 170(3):615-21. (IF: 2.15)

[3] Tayebi N, Jamsheer A, Flöttmann R, Sowinska-Seidler A, Doelken SC, Oehl-Jaschkowitz B, Hülsemann W, Habenicht R, Klopocki E, Mundlos S, Spielmann M. **Deletions of exons with regulatory activity at the DYNC1I1 locus are associated with split-hand/split-foot malformation: array CGH screening of 134 unrelated families.** Orphanet J Rare Dis. 2014 Jul 29; 9(1):108. (IF: 3.358)

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