Gene expression profiling and gene regulation for functional genomics in mouse models

Habilitationsschrift

vorgelegt von

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CONTENTS

1. SUMMARY

Mammalian genes have multiple functions in time and space during the development of the organism, during aging, and in health and disease. This phenomenon of pleiotropic gene function is a major factor contributing to the drastic increase of complexity from the mere number of approximately 25.000 protein-coding genes in the mammalian genome to the level of the organism. When and where a gene is expressed is an integral part of gene function. The profiling of gene expression and the study of gene regulation are therefore intriguing scientific objects of current biology.

For a systems level approach to measure changes in transcript profiles in tissues of mouse models for human diseases we have used microarray (DNA-chip) technologies. Data from more than 1.100 microarray experiments and 46 mutant mouse lines have been analysed and complemented with comprehensive phenotype data from the German Mouse Clinic (chapter 3.1.). The integration of multiple level information, including transcriptome, proteome, and metabolome is a major corner stone in renewed efforts to undertake systems biology approaches (chapter 3.2.). We have performed a comparative analysis of mouse kidney and liver transcriptomes and proteomes. This provided the proof-of-principle that such studies are feasible in the mammalian organism despite the complexity of tissues. The combination of transcriptomics, proteomics and quantification of metabolites allowed us to dissect the specific requirement of a transmembrane peptide transporter in renal physiology. Finally, we applied the expression-profiling approach to the identification of new components of the *Delta-like 1* (*Dll1*) signal during mouse embryonic development (chapter 3.3.). Novel *Dll1* targets are now object of functional studies in gene targeting and mutagenesis approaches in the mouse. The *cis*-regulation of the *Dll1* gene in the mesoderm of the developing embryo is studied in transgenic and mutagenic approaches towards a better understanding of the mechanisms that act on gene regulation in mammalian cells in a specific example.

2. INTRODUCTION

Gene expression-profiling and gene regulation for functional genomics in mouse models.

2.1. Positioning of past and current research in the field.

Why study gene expression?

Comparative genomics of the mouse and human genome draft sequences estimated a protein-coding gene count of approximately 30.000 genes for both species (Waterston et al., 2002). With the 99% completion of the human euchromatic genome sequence this estimate declined to 20.000 to 25.000 proteinogenic loci (IHGSC, 2004). This rather unexpected low number of coding genes has generated a seeming paradox: The complexity of the mammalian Bauplan, cell differentiation and patterning processes during embryogenesis, the organism's physiology and its highly integrated response to (novel) biotic and abiotic environmental factors may not be sufficiently explained by the mere number of genes. This situation is reminiscent of the earlier *C-value paradox* that there is no significant correlation between the amount of eukaryotic, nuclear DNA and complexity of the organism (Cavalier-Smith, 1978).

2.2. Gene regulation increases genome complexity.

A theoretical consideration.

Despite the fact that there is no precise definition of the term *complexity of the organism* (Emmeche, 1997), genetic complexity is dramatically increased at the protein level and through the spatial and temporal control of gene expression. Considering only the simplest model in which complexity (c) would be described by simple on and off states (x = 2) of gene transcription, a difference of, for example, a few hundred genes (e.g., n = 500) in the total gene count between two species would suffice to stretch out a theoretical transcriptome space of astronomic dimensions ($c = x^n = 2^{500} \approx 3.3 \times 10^{150}$; compare this number to the estimated count of 10^{80} atoms in the observable universe). The simple mathematical example does not account for the reasonable assumption that an unknown number of transcriptome states may be lethal and is obviously hypothetical. However, it illustrates that the believe that a total count of 20.000 to 25.000 genes in the human genome would be unexpectedly low or insufficient to

account for mammalian complexity is not justified. Gene regulation is one important factor that increases genetic complexity dramatically and is, therefore, an intriguing scientific object. The impression that the mammalian set of genes appears to be small rather may reflect a largely incorrect perception of what gene products are. If gene products are considered merely as building blocks or *parts* of the cellular machinery then a living organism may be regarded less or similarly complex as a modern airplane, assembled from 200.000 unique parts, each of which directly interacts with three or four others on avarage (Claverie, 2001). Instead, if gene products are thought of as *tools* (for chemical modification) that construct and maintain the living cell and the mutlicellular organism, then complexity again dramatically increases.

2.3. Gene products as multi-functional tools.

Gene regulation as integral part of gene function.

Gene products often have distinct functions at different developmental stages, during aging or in different cells in the organism. Ludwig Plate commented already in 1913 that the multi-functionality of *Mendelian factors* (genes) would argue against a high number of genes (cited in (McKusick, 1976)). In his inaugural speech as Haeckel's successor in the chair of zoology at the University of Jena, he introduced and defined the term *pleiotropism* as description for the multifunctionality of genes (Plate, 1910):

" ... Pleiotrop nenne ich eine Einheit, wenn von ihr mehrere Merkmale abhängen, die dann natürlich stets zusammen auftreten und daher als korrelativ gebunden erscheinen. Je mehr die Mendelschen Forschungen sich vertieft haben, desto mehr Beispiele sind bekannt geworden, die sich nur unter der Annahme pleiotroper Faktoren verstehen lassen. ...".

The concept of pleiotropism subsequently became of great interest to the study of gene function. One important problem to its experimental study was the distinction between *genuine* pleiotropism, where a gene has more than one primary effect, and *spurious* pleiotropism, where a gene has one primary function that ultimately affects multiple phenotypic traits (Gruneberg, 1943). One of the earliest experimental proofs for genuine pleiotropism in a mammalian organism was provided by a detailed analysis of the *W* (*white spotting*) mutation in the *Kit* oncogene (Hayashi et al., 1991) and the *f* (*flexed tail*) mutation in a yet

unidentified gene (contradictory reports: (Fleming et al., 2001; Lenox et al., 2005)). Both alleles affect the size and number of erythrocytes, and intensity and location of hair pigment. The enduring and meticulus analysis of epistasis and phenotypes of the *W* and *f* alleles led to the conclusion that both the ventral white spotting and the anemia each resulted from genuine, primary functions of the mutated genes (Russell, 1949; Russell and McFarland, 1966). Although a comprehensive assessment is currently not available, browsing through the annotated mammalian genome strongly suggests that highly multifunctional genes are rather the rule than the exception.

Spatial and temporal control of gene regulation is thus a prerequisite for pleiotropic gene function. Accordingly, it was demonstrated that the *Kit* gene during embryonic development is transcribed in migratory cell lineages such as melanocytes, primordial germ cells and hematopoietic stem cells, all of which are affected by mutations in the *Kit* gene (Keshet et al., 1991; Motro et al., 1991; Orr-Urtreger et al., 1990). In addition, reporter gene expression from the *Kit* locus (*Kit*^{tm1Alf}, synonym: *Kit*$^{W\text{-}lacZ}$) suggested that during embryogenesis *Kit* is not only expressed in cells that require its function, but also in cells that are - according to current knowledge - not affected by mutations in *Kit* (Bernex et al., 1996). However, ectopic expression of *Kit*, for example from the *Kit*^{Wsh} allele, also affected early melanogenesis and was responsible for pigment deficiency (Duttlinger et al., 1995; Duttlinger et al., 1993).

Due to its historical significance the *Kit* gene was selected here to illustrate the interdependence of gene function and transcriptional regulation using a classical example for a pleiotropic mammalian gene. Findings made with this gene may be generalised for the majority of genes in the mammalian genome. Transcriptional expression is a condition *sine qua non* for a gene product to function. Genes and their products can have different functions in different cells or at different time points. A gene may be expressed in cells where it is apparently not required. However, ectopic expression of a gene may also result in detrimental mutant phenotypes. Thus the spatial and temporal control of transcriptional regulation is integral to pleiotropic gene function.

2.4. The presented research in a nutshell.

From systematic expression-profiling to single gene functional approaches.

The study of gene expression, in particular at the genomic scale, is an intriguing scientific object due to the reasons explained above. Thus a major focus in the research projects that are presented in this script is the analysis of gene expression at the transcript and protein levels. Several of these research projects are centred on the use of the DNA microarray technology for genome-wide gene expression-profiling mostly of coding RNAs in tissues of mouse mutant lines.

The first chapter (3.1.) deals with establishing a platform for the systematic gene expression-profiling of mouse tissues. A brief introduction to the advent of DNA microarrays for the profiling of transcriptomes and to the technology itself will be given. The presented research data in this chapter deals with the experimental control of hybridisation specificity on the microarray, the proof-of-principle for the usability of microarray based transcriptome profiles for molecular phenotyping, and the application of the method for the identification of regulated target genes in complex mammalian tissues and in vitro models.

In the second chapter (3.2.) work is presented that aims at molecular profiling beyond the transcript level. Comparative analyses of transcriptomes and proteomes of mouse tissues were made, and co-expressed and co-localizing genes in the mouse genome were identified. The profiling of transcripts, proteins and metabolites in kidneys of mice deficient for a peptide transporter revealed a coherent molecular network pointing to the functional requirement of the mutated gene. Since the research summarized in this chapter should be seen in the context of current attempts to develop algorithmic models of more holistic, molecular interactions, a short introduction to systems biology is included.

In the third chapter (3.3.) research projects are summarized that focus on the functional analysis of Delta/Notch related signalling during somitogenesis in mice. Expression-profiling and *in situ* hybridisations were combined to screen for regulated genes in *Dll1* (*Delta1*, *Delta-like 1*) deficient embryos. This has led to functional studies of novel *Dll1* target genes during somitogenesis. In transgenic mice the requirement of restricted expression of the *Dll1* gene for the proper development of the vertebral column from embryonic somites was examined. To begin to understand the *cis*-regulation of *Dll1* the functional requirement of a mesodermal enhancer was analysed. Since somitogenesis and Delta/Notch

signalling are universal among vertebrates a brief introduction to the biological process will be given in the beginning of this chapter.

A short section follows each of the three chapters on perspectives, which include ongoing projects and own unpublished data. The main intention of this script is to present the work that has been published during the habilitation in a coherent framework. To avoid redundancy, details of the published data, and materials and methods are not repeated, and can be found in the attached reprints of peer-reviewed publications.

3. RESEARCH

3.1. MOLECULAR PHENOTYPING OF MOUSE TISSUES USING MICROARRAY BASED TRANSCRIPT PROFILING.

Introduction

3.1.1. DNA-microarrays for gene expression-profiling.

From the emergence of the technology to its clinical application.

Gene expression analyses have been performed on a gene-by-gene basis since the mid seventies typically using Northern blot analyses (Southern, 1975). Since the development of an efficient method for the amplification of DNA fragments in the mid eighties reverse transcription PCR has become the most sensitive method for the detection of expressed genes (Saiki et al., 1985).

For the simultaneous analysis of a large number of transcripts in a single experiment two methods, serial analysis of gene expression (SAGE) and DNA microarrays, are used today. SAGE is based on the theoretical consideration that short sequence tags of 10 to 14 basepairs should be sufficient to identify the gene from which the cDNA tag has been isolated (Velculescu et al., 1995). The frequency with which such SAGE tags are identified provides the expression level of the corresponding gene. To obtain this information SAGE tags are isolated from RNA pools, concatenated, cloned and finally sequenced. This technology has been used for the evaluation of differential transcript profiles, for example, during vertebrate embryogenesis (Logan, 2002; Tan et al., 2002), in diseased tissues and for the identification of potential drug targets (Boheler and Stern, 2003; Porter and Polyak, 2003; Porter et al., 2006; Ye et al., 2002). SAGE does not require any a priori knowledge on expressed sequences. It can detect novel genes in the transcriptome, provided that the SAGE tag is from a unique position within the expressed transcript (Wang, 2007). LongSAGE and SuperSAGE are adaptations of the original method to extend the length of SAGE tags (Matsumura et al., 2003; Saha et al., 2002). Although these longer tags increase the specificity of SAGE tags to represent a single transcript or gene, their mapping rates are markedly decreased. This is likely due to an increased probability of incorporating base errors in long tags as compared with short tags.

Whereas SAGE is a sequencing-based method, microarray gene expression-profiling is a hybridization-based technology. It is more commonly used than

SAGE, possibly because the latter is technically more challenging and the interpretation of data is more difficult. Microarrays for expression-profiling use a solid support to immobilize specific probes in defined positions. These microarrays are then hybridized to a complex mixture of expressed nucleic acids that are most commonly radioactively or fluorescently labelled. This is in contrast to Southern or Northern blot analyses where the complex mixture of expressed genes is immobilized on a membrane and hybridized to one specific and labelled probe.

The first publication in which the concept of microarrays for gene expression-profiling was originally applied (Augenlicht et al., 1987), unfortunately is rarely cited in reference to this technology. In this paper nylon membranes containing the hitherto amazingly high number of 4000 cloned cDNAs was used to profile relative gene expression in normal and neoplastic human large intestine biopsies and in colonic carcinoma cells differentiated in vitro. Possibly because the authors focused on their biological questions rather than on the application of a novel technique, this publication is rarely recognized as the first application of microarrays. The most commonly used method for expression-profiling microarrays, in particular for custom-made arrays, employs glass as solid support for probes (*DNA-chips*), and fluorescent dyes as label for the complex target mixture. A major advantage of this approach is the fact that it allows dual-color, competitive hybridizations on a single DNA-chip. This greatly facilitates the direct comparison of expression levels in an experimental and a reference sample, since it is not required to ascertain perfect chip-to-chip reproducibility of spot size and spot morphology, and the absolute amount of probe contained in a spot. Custom-made DNA-chips are generally printed on slides, the size of a normal histological slide. In contrast to membranes this allows a great deal of automation in spotting, hybridization washing, and scanning. The proof-of-principle for this DNA-chip strategy was provided using poly-L-lysine coated glass slides as support for 48 PCR amplified cDNA probes (Schena et al., 1995). This DNA-chip was used to estimate the dynamic range of the detection method, confirm the regulation of specific genes in transgenic Arabidopsis thaliana, and between root and leaf tissues. The feasibility to extend the technology to genome-wide expression-profiling approaches was already anticipated in this publication. Dual-color, competitive DNA-chip hybridizations are still most commonly used for

microarray expression-profiling with cDNA microarrays that are produced in academic laboratories. Commercial DNA-chips often use oligonucleotide probes that can also include mismatch probes in addition to the perfect match probes. Such oligos may be manufactured by in situ synthesis on glass in a combination of photolithography and oligonucleotide synthesis chemistry (Pease et al., 1994). Alternatively, long oligonucleotide probes can be attached to beads and distributed on a micro-waver like solid support. Such commercial arrays can be produced with a chip-to-chip reproducibility and density that is difficult to obtain by a spotting method. The reproducibility of these DNA-chips is generally good enough that it is no longer favourable to perform competitive, dual-colour hybridisations. Instead, a single set of reference sample experiments is sufficient to analyse expression profiles of more than one experimental sample, strongly reducing the amount of reference RNA needed in more complex experimental set-ups. A multi-site consortium, the MicroArray Quality Control (MAQC) project, has demonstrated the high correlation coefficients and high concordance of differentially expressed gene lists between the one- and two-colour approaches (Patterson et al., 2006). The MAQC project also analysed the correlation of microarray data in relation to alternative quantitative PCR-based platforms (Canales et al., 2006), as well as the consistency across different microarray platforms and across different user sites (Shi et al., 2006).

Today a plethora of papers has successfully used expression-profiling to molecularly classify tumour types and groups of patients, predict clinical outcome upon treatment, identify affected pathways and novel genes involved in biological processes and to suggest targets for therapy. These findings from retrospective studies have generated a great interest in the application of microarray-based expression-profiling to human samples in real-time clinical studies (Lemmer et al., 2006). Despite these great expectations, DNA-chip expression-profiling still has to find its way to clinical diagnostic applications (Ji and Davis, 2006). However, a number of chip designs for expression-profiling are at least in clinical trials (Burczynski et al., 2005; Haferlach et al., 2007; Staal et al., 2006). Besides its application for gene expression analysis, DNA-chip based technology has been approved for a clinical diagnostic purpose in the US and the European Union. This DNA-chip identifies allelic variations of two cytochrome P450 genes that determine the metabolic rates of several frequently prescribed drugs. This

apparently first chip-based clinical diagnostic test aids physicians in selecting drugs and individualizing treatment dosis according the patients genotype. Presumably, the line of chip-based diagnostic products will grow in the coming years, including also expression-profiling applications.

3.1.2. A story of success.

Application of microarray based expression-profiling for mouse tissues.

Searching the MEDLINE database for publications with the terms *microarray* and *expression-profiling* in 2006 retrieves close to 2100 records. Restriction of the search to *mouse* still identifies 419 publications in 2006, 356 in 2005, 311 in 2004, 215 in 2003, 150 in 2002, 60 in 2001, 12 in 2000, 2 in 1999, and 1 in 1998. No attempt will be made here to provide a comprehensive summary of this literature. Instead some of the most significant, early publications using microarrays for transcript profiling in mice will briefly be introduced.

In one of the earliest microarray based expression-profiling studies, it was shown that ageing in mice is associated with changes in the expression of genes in brain and muscle that are indicative of inflammatory response, oxidative stress and reduced neurotrophic support. These ageing related gene expression patterns were at least partially reversed by caloric restriction, suggesting that metabolic alterations have profound effects on brain ageing. The transcriptional response to ageing in the mouse brain had significant similarities to that in human neurodegenerative disorders, such as Alzheimer's disease (Lee et al., 1999; Lee et al., 2000a).

The differential gene expression in several brain regions and the response to seizure have also been analyzed and the results provided evidence that differences in gene expression may contribute to behavioural differences between mouse inbred strains (Sandberg et al., 2000). This and other early microarray studies in mice (Campbell et al., 2001; Livesey et al., 2000; Porter et al., 2001) have provided the proof-of-principle that despite the complexity of mammalian organs, expression-profiling is a useful tool to identify pathways associated with particular biological processes in the mouse model system.

At the same time data from distinct regions of the mouse brain apparently suggested an inverse correlation between tissue complexity (as based on the number of cell types) and the number of regulated genes (Sandberg et al., 2000).

Whereas low-abundance transcripts that are expressed at 1 copy in 100.000 may be detected in microarray hybridisations of single cell type preparations, these may no longer be identified in more complex biopsies that comprise 10 or 100 different cell types. Thus, one possible interpretation of the inverse correlation from brain regions of different complexity is that the less complex the tissue being studied, the more likely expression differences will be identified (Geschwind, 2000). Accordingly, the combination of laser-microdissection (Schutze and Clement-Sengewald, 1994; Schutze and Lahr, 1998) with the microarray hybridisation technique has proven as one important method for the detection of differentially regulated low abundance transcripts (Aoyagi et al., 2003; Feldman et al., 2002; Klur et al., 2004; Mills et al., 2001; Xiang et al., 2003).

Own research

From proof-of-principle to molecular phenotyping in the German Mouse Clinic.

3.1.3. Establishing a high quality microarray platform for gene expression-profiling.

With the aim to use genome-wide gene expression-profiling as a tool for the molecular phenotyping of mouse mutants and models for human diseases, we established a microarray platform (Beckers et al., 2002). Microarray expression-profiling is a complex experiment consisting of several consecutive steps: fluorescent or radioactive labeling of extracted RNA, hybridisation, washing, scanning, image processing and data analysis. Each of these steps is a potential source of artefacts that may bias the result and the exact appraisal of differential gene expression. In parallel, the quality of expression-profiling experiments also depends on the design and quality of the arrays themselves. A series of decisions were made to identify the type of platform that would best suite the requirements of a high-quality and medium-throughput (20 to 40 DNA-chips per week) platform in our academic research environment.

We first decided on the probe sequences to be arrayed. In general, sequences for probes can be identified, for example, from the public UniGene database (Wheeler et al., 2003). This database automatically partitions GenBank sequences into a non-redundant set of gene-oriented clusters. Each UniGene cluster contains sequences that represent a unique gene, as well as related

information such as the tissues in which expression of this gene has been detected in previous experiments and the chromosomal map location. For Mus musculus the UniGene database (build #161) contains approximately 4,1 million sequences in clusters and close to 19.500 clusters that contain both mRNAs and expressed sequence tags (ESTs). UniGene clusters in most cases contain several clones that could be selected as probes for microarrays. As with other hybridization methods such as Southern blot analyses or in situ hybridization methods the choice of the best working probe requires careful consideration. The probe selection should include criteria such as uniqueness of the sequence, content of GC and AT bases, hypothetical melting temperature and so on. Based on these considerations we decided to use the mouse arrayTAG clone set (Lion Bioscience AG). This clone set comprises more than 20.000 non-redundant cDNA fragments that are mostly derived from 3'UTRs (untranslated regions) of mRNAs. All 20.000 probes have been sequenced and the bacterial stocks are of high quality. Having the sequence allows to blast each probe over the most recent up-date of the mouse genome sequence and to verify the uniqueness of the probe. Whereas synthesized oligonucleotide probe sets have the advantage that they can be spotted on arrays immediately, cDNA probe sets have the benefit that they are a regenerative source. We amplify our clone set by PCR from bacterial lysates, followed by the purification and pooling to obtain the required concentration and quantity of the probe. Each amplified probe is tested on agarose gels to validate the quality of the PCR product. Using a liquid handling robot the amplification of the bacterial probe set was automated to a large extend.

PCR products are subsequently spotted by a robotic microarrayer (BioRobotics MicroGrid) onto glass slides (chips). The production of microarray membranes (nylon or nitrocellulose) is technically less challenging. However, membranes may contain only several hundred probes, and are not well suited for an automation of hybridization and scanning processes. In addition, glass as transparent, solid support allows the simultaneous, competitive hybridization of test and reference samples labeled with distinct fluorescent dyes. Relative expression levels are analyzed directly by comparing both fluorescent signals on a feature. Glass chips and membranes can be purchased with surfaces that have been pretreated to augment their surface charge, to increase the adherence of

the probe, and to reduce background signals (Schulze and Downward, 2001). In our case, we obtained best results in terms of spot size and shape, and signal strength using aldehyde coated slides that are cross-linked to amino-tagged PCR-amplified probes (Figure 1).

We use a hybridization station (Tecan HS4800) to automate the parallel hybridization of several DNA-chips, their washing and drying. This is helpful to improve the chip-to-chip reproducibility of signal intensities.

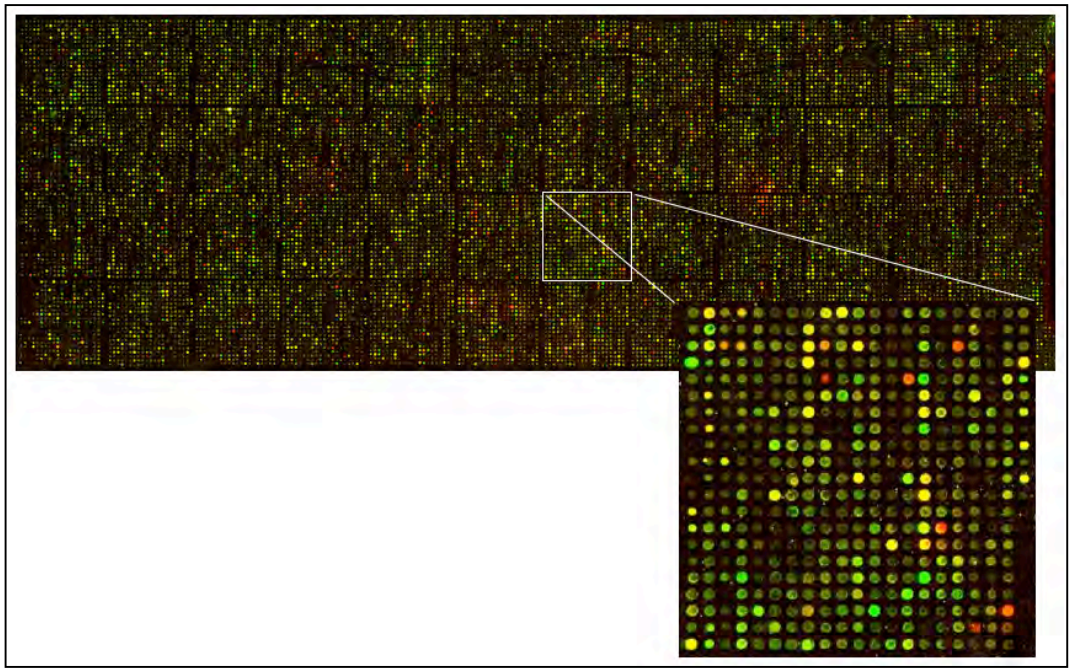

Figure 1: *Example of a dual color hybridisation with cyanine dye (Cy3 and Cy5) labeled cDNA from two mouse tissues on our DNA-chip displaying the 20.000 mouse arrayTAG clone set.*

3.1.4. A novel method to experimentally assess the specificity of nucleic acid hybridisation in situ on the microarray surface.

The reliability of expression profiles obtained in DNA-chip experiments is a major concern for the exact appraisal of differential gene expression (Knight, 2001). The repetition of experiments and replicates of clones in an array (Lee et al., 2000b; Tseng et al., 2001) are standard procedures often used to support the reliability of expression data. However, such procedures cannot exclude the generation of false data. Artefacts can be due, for example, to particular probe sequences and secondary structures that cause cross-hybridisation. Such false

data may therefore be highly reproducible (Drobyshev et al., 2003a). Another approach is the use of multiple probes for the same mRNA. The number of such probes for one specific gene may be as high as 40 in commercial microarrays (Li and Wong, 2001). This strategy requires a high number of specific oligonucleotides per gene, is expensive, and relies on the assumption that the majority of probes for each gene produce specific hybridisation, which is not valid a priori. Therefore, we were seeking a method to verify the quality of each individual probe immobilised on an array in relation to the target RNA used for hybridisation. Although we applied this technology to tissues from mouse lines, this procedure may equally be applied to biological material from other sources.

It has been shown that melting of dsDNA in solution can be described as a melting curve with sigmoidal shape (Voet and Voet, 1995). In such experiments it was proven that for specified solutions the melting temperature depends on the DNA sequence and is maximal for full-length perfect matches. Thus, it is possible to assess the extent of specific hybridisation and cross-hybridisation by measuring melting curves over increasing hybridisation or washing stringencies. In some early applications of microarray technologies it was pointed out, that such “melting curves could provide an additional dimension to the system and allow differentiation of closely related sequences” (Stimpson et al., 1995). Subsequently, similar methods were used for mutation diagnostics in the beta-globin gene (Drobyshev et al., 1997), for the determination of on-chip DNA duplex thermodynamics (Fotin et al., 1998; Kunitsyn et al., 1996), and for the highly parallel study of DNA interactions with low molecular weight ligands (Drobyshev et al., 1999) and proteins (Krylov et al., 2001). However, this principle had until then not been applied to the most popular application of microarrays, the expression-profiling technology, using DNA-chips.

We used this method to examine probe specificity on our custom made DNA glass chip in combination with different pools of target sequences isolated from different mouse tissues. Melting curves of hybridised targets were measured after repeated washing of microarrays with increasing stringencies and the recording of the hybridisation signal intensity for each array element at each step. In case there are different fractions of target hybridised to the same probe, these will be washed off from the array at various stringencies due to different extends of double strand formation. The set of such data for each array element comprises

the fractionation curve, which provides novel information that can be used to evaluate hybridisation data reliability. The iterative use of this approach improves the selection of gene specific probes for DNA microarray experiments based on experimental data and will thereby optimize the overall performance of DNA microarrays. The approach confirmed the high quality of our custom made mouse DNA-chip. Based on the melting curves measurements we estimated the portion of specific hybridisation signals to at least 98,3% depending on the tissues analyzed (Drobyshev et al., 2003b).

3.1.5. Systematic RNA expression-profiling of mouse mutants. Identification of affected organs in ENU induced mutant lines.

The feasibility to monitor genome-wide gene expression levels allows an unbiased way to assess molecular phenotypes that may result, for example, from a mutation, a “knock-out” or a transgene insertion. We expected that such highly parallel expression studies might detect phenotypes at the molecular level that may otherwise not be detected in phenotypic screens that specialise, for example, on dysmorphologies, selected blood, metabolic, immunological or behavioural parameters (Beckers et al., 2002). To address this question, we used a systematic DNA-chip based expression-profiling approach to identify affected organs in ENU induced mouse mutant lines (Beckers and Hrabe de Angelis, 2002). At that time, systematic approaches in vertebrates had been performed only to analyse gene expression profiles for organ specific expression in mice and to molecularly phenotype mutagenised mouse embryonic stem (ES) cells (Bono et al., 2003; Matsuda et al., 2004; Symula et al., 2004), but not for the analysis of series of mouse mutant lines. As a prerequisite to the approach, we found in general low numbers of differentially expressed genes in organs from ENU-induced mutant lines, in particular, when individual RNA and not pooled samples were used in microarray hybridisations (Seltmann et al., 2005). This finding opened the possibility to use expression-profiling as screening tool for the identification of transcriptionally affected organs in mutant mice. One out of 8 organs from 5 different mutant lines yielded a set of differentially expressed genes not found in any other mutant line tested. The identified regulated genes were verified by real time PCR.

More than 200 DNA-chip experiments had to be performed to identify one affected organ in ENU mouse mutants. It was suggested that a combination of an increased number of experimental samples and sub-pooling strategies could improve the throughput in microarray experiments (Peng et al., 2003). Augmented numbers of differentially expressed genes in pooling strategies as compared to individual sample hybridisations have been reported (Agrawal et al., 2002). To assess the validity of expression data from pooled samples we performed a direct comparison of data obtained from identical samples in pooling and non-pooling strategies. Genes consistently up-regulated in one mutant line were also detected with the same tendency in the pooled RNA sample, although these genes did not meet the more stringent criteria of significance. However, the majority of genes detected in pooled samples from the same RNA isolations were false positives due to strong over-expression of some genes in individual mice (Seltmann et al., 2005). Several of these false positive genes were also found in hybridisations of individual wild-type samples against each other. These observations suggested that, if biological variation in gene expression levels is an issue, pooling of samples is not an optimal method for the identification of differential gene expression. We also provided evidence that controlling variables such as stress, social status, or food intake before organ sampling reduces biological noise in gene expression levels.

3.1.6. Gene expression-profiling as molecular phenotyping method.

The molecular phenotyping screen in the German Mouse Clinic.

Following the proof-of-principle in ENU induced mouse mutant lines, we extended the molecular phenotyping screen to mouse models that are generated by gene targeting, gene trap, transgenic insertion or any other type of mutagenesis. For this purpose, the gene expression-profiling screen was integrated as one of 14 phenotyping screens in the German Mouse Clinic (Gailus-Durner et al., 2005). Within this open platform for systematic and comprehensive phenotyping of mouse mutant lines, we have chosen a semi-biased approach: Organs of mouse mutant and reference strains are systematically archived in quintuples. For gene expression-profiling organs are selected based on either conspicuous phenotypes in other screens of the

German Mouse Clinic (GMC) or based on previous knowledge on the mutated gene.

Until now the GMC core facility has provided 61 mutant mouse lines and 8 wild-type strains for analysis in the molecular phenotyping screen. Initially, a set of 17 organs was routinely archived for the primary screen. This set of organs was subsequently modified such that organs that were never requested for gene expression-profiling were transferred to the secondary screen. Other organs, such as adipose tissues, that were requested occasionally for expression-profiling were added to the primary screen. In total, 10.456 organs of 744 animals have been collected to date (Horsch et al., manuscript in preparation).

Either based on conspicuous phenotypes in other GMC phenotyping screens or based on previous knowledge of gene functions, 90 organs of 46 different mouse mutant lines have been selected for gene expression-profiling (Table 1). For 68 of the 90 organs the selection was based on mutant phenotypes in other GMC screens. For the remaining 22 organs, the selection was based on previous knowledge about the mutated gene. For example, mutant phenotypes that were detected in either the behavioural or neurological GMC screens often led to the choice of brain or muscle as organs for gene expression analyses. Changes of immunological parameters were used to select spleen or thymus and so on (Table 1)

Total RNA for expression-profiling was isolated from 988 individual organs and used for 1152 cDNA-chip experiments (Horsch et al., manuscript in preparation). These numbers include only chip experiments that were technically acceptable. Initially four technical replicates were performed with individual RNA preparations against an RNA-pool of wild-type mice. Subsequently the number of experimental replicates was reduced to a singly dye-swap pair, since additional technical replicates did not significantly improve the information on regulated genes.

Using the TIGR software suite, TM4 (Saeed et al., 2003), including MIDAS for normalization (Quackenbush, 2002) and SAM for identification of genes with significant differential regulation (Tusher et al., 2001), we identified regulated genes in 45 of 90 analysed organs (Table 1). The 90 organs that were analysed for differential gene expression were collected from 46 distinct mouse mutant lines. Differential gene expression was detected in 25 of the 46 mouse mutant lines (Horsch et al., manuscript in preparation). Thus, the efficiency of detecting

regulated gene expression in relation to the overall number of microarrays experiments strongly increased in the semi-biased approach used in the GMC (43 organs in 1152 chip experiments (1:28)) as compared to the previous systematic screen of ENU induced mutant lines (1 organ in 160 chip experiments (1:160)).

In order to analyse whether particular organs display stronger differential gene expression than others, we analysed the mean fold induction/repression of gene expression combined from all mutant mouse lines with positive results. Whereas the mean fold regulation in organs such as muscle, spleen, testis, and heart was around two-fold, mean regulation in kidney and liver was above four-fold.

In the 90 analysed organs, differential gene expression was detected for 1251 of the ~21.000 probes on our cDNA microarray (Horsch et al., manuscript in preparation). Several of these genes are regulated 2-3 times in the same organ of different mutant lines (Table 2). For example, in spleen 17 genes with differential gene expression in two to three different mutant strains and in liver 21 genes regulated in two mutant lines were found. One potential reason for this finding could be that the selection of the organ is based on similar findings in identical phenotypic screens.

However, regulated genes were detected also across different organs. 261 genes were differentially expressed in up to 8 different organs (Table 3). These genes are predominantly associated with structural molecule activity (e.g. genes of the ribosomal protein family), iron ion binding (haemoglobin, cytochrome and ferritin) and RNA binding (splicing factors, RNA binding proteins). *Glutamine synthetase* (*Glul*) was overexpressed in eight mouse mutant lines. Changes of expression levels in six different tissues were found for *CR516145* and *Perq1* (Table 3). Nine and 13 genes were regulated in five and four mutant lines, and 61 and 175 genes were significantly differentially expressed in three and two mouse mutant strains. While *CR516145* is up-regulated and *Perq1* down-regulated in 5 of 6 different mutant lines, for the other genes no clear tendency in terms of up- or down-regulation could be detected.

As part of the Eumorphia consortium we contributed to the development of the European Mouse Phenotyping Resource for Standardized Screens (EMPReSS) that were validated across several phenotying centers in Europe (Brown et al., 2005).

organ	times selected	regulated genes		number of regulated genes	GMC screens with mutant phenotype
		yes	no		
brain	20	10	10	1 - 100	behavior (19) neurology (7) nociception (6) pathology (2)
spleen	14	8	6	3 - 85	immunology (9) pathology (5) allergy (1) clinical chemistry (1) previous knowledge (5)
liver	11	5	6	5 - 232	energy metabolism (7) clinical chemistry (4) previous knowledge (2)
thymus	10	7	3	7 - 77	immunology (6) pathology (3) allergy (1) clinical chemistry (1) previous knowledge (4)
muscle	10	6	4	2 - 72	neurology (9) behavior (1)
kidney	6	1	5	61	clinical chemistry (5) pathology (1) previous knowledge (1)
heart	5	1	4	19	cardiovascular (1) previous knowledge (4)
testis	5	2	3	3 - 215	pathology (2) previous knowledge (3)
bone	3	2	1	23 - 197	dysmorphology (3)
lung	3	1	2	20	lung function (3)
auricle	1	0	1	0	previous knowledge (1)
fat white	1	1	0	30	previous knowledge (1)
fat brown	1	1	0	72	previous knowledge (1)
salivary gland	0	–	–	–	–
bulbourethral gland	0	–	–	–	–
seminal vesicles	0	–	–	–	–
eye	0	–	–	–	–
total	**90**	**45**	**45**		

Table 1 *Efficiency of gene regulation detection in the semi-biased GMC molecular phenotyping screen.*

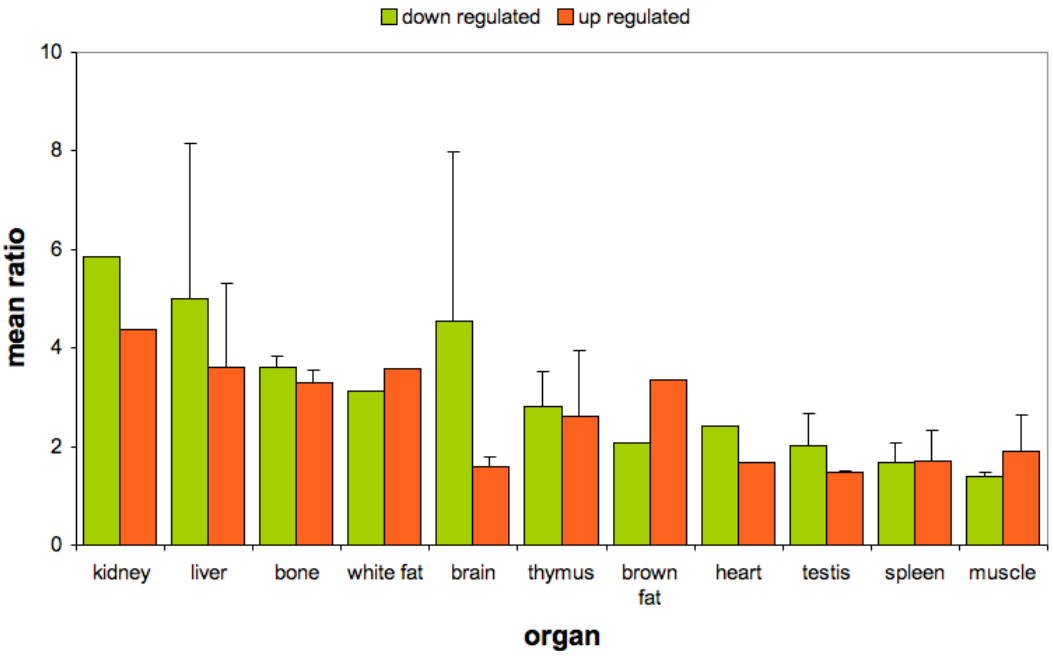

Figure 2: *Combined mean fold repression and induction of gene expression in all mutants with differential gene expression.*

organ	number of genes regulated in more than one mutant line	number of mutant lines in which these genes were regulated	number of times the analysis of this organ detected differential gene expression
liver	21	2	5
spleen	17	2 or 3	8
muscle	15	2	5
thymus	12	2 or 3	7
brain	7	2 or 3	9
bone	4	2	2
testis	1	2	2

Table 2: *Number of genes regulated in a particular organ in more than one mutant line.*

frequency	number of genes with that frequency	gene symbols or common gene functional annotation
8	1	Glul
6	2	CR516145, Perq1
5	9	2210411K11Rik, Actb, Blvra, Herpud1, Ldha, Lyzs, Mark3, Rps11, Scd1
4	13	1300018I05Rik, 5730419I09Rik, Arbp, Atp5h, Grrp1, H3f3a, Hba-a1, Ly6e, Mfi2, Nup155, Rhob, Snag1, Uba52
3	61	structural molecular activity, RNA & iron ion binding
2	175	
total	**261**	

Table 3: *Genes detected as regulated across all mouse mutant lines analysed in the GMC molecular phenotyping screen.*

3.1.7. Molecular phenotyping of in vitro systems

Manuscripts on the functional analysis of individual mouse mutant lines, which include data from the molecular phenotyping GMC screen, are currently being prepared for publication. In addition, we have used the DNA microarray platform for the transcriptome analysis of in vitro systems. Two published examples, one for the study of the transforming activities of the *ERBB2* oncogene and the second for the effect of Scrapie protein infection on transcription profiles in different neuronal cell lines are summarized below to describe the biological information that may be gained from genome-wide transcript profiles.

Identification of novel ERBB2 target genes:

The protooncogene *V-erb-b2 erythroblastic leukemia viral oncogene homolog 2* (*ERBB2*, *HER2*, *NEU*) encodes a 185 kDa transmembrane glycoprotein with tyrosine kinase-specific activity and extensive homology in structure and sequence to the *epidermal growth factor receptor* (*EGFR*). *ERBB2* is expressed in a variety of normal tissues but amplified and/or overexpressed in 20 - 30 % of breast, ovarian and renal cell carcinomas (Brossart et al., 1998; Seliger et al., 2000; Slamon et al., 1987). Its up-regulated expression is often associated with a rapid disease progression, chemoresistance of tumours as well as poor prognosis of patients (Brooks et al., 2003; Cleator et al., 2002). Due to its oncogenic capacity, *ERBB2* has been used as a target for anti-tumour therapy,

including treatment with the humanized monoclonal antibody (mAb) Herceptin® (Trastuzumab, Genentech, Inc.) and T cell-based immunotherapies both in animal models and humans (Kiessling et al., 2002; Quaglino et al., 2004; Shawver et al., 2002). A complete eradication of *ERBB2* overexpressing tumours requires a combination of humoral and cellular *ERBB2* targeted immune responses. Since the response rate of these immunotherapies is still limited, novel therapeutic markers and targets are urgently needed for the treatment of patients with *ERBB2* overexpressing tumours (Fischer et al., 2003).

The oncogenic transformation of cells is characterized by complex changes in gene expression at the transcript and protein levels (Louro et al., 2002; Zuber et al., 2000). So far, only a small number of signal transduction pathways regulated by *ERBB2* has been characterized (Bertucci et al., 2004; Kauraniemi et al., 2001; Mackay et al., 2003; Wilson et al., 2002). Even less is known about transcriptionally regulated target genes at a genome wide scale that contribute to the tumorigenic effect (Hernan et al., 2003; Neve et al., 2002). A panel of *ERBB2* overexpressing breast carcinoma cell lines was previously examined using DNA microarrays (Kumar-Sinha et al., 2003). In this study, a limited set of *ERBB2* regulated genes, in particular genes associated with increased fatty acid synthesis was described. DNA-chip based expression-profiling was also applied to study the co-regulation of genes in the vicinity of the human *ERBB2* gene following DNA amplification (Kauraniemi et al., 2001).

In our study we extended the analysis of *ERBB2* dependent gene regulation using our own mouse DNA microarray described in chapter 3.1.3 to analyse differential gene regulation in *ERBB2* transfected versus wild-type (wt) NIH3T3 cells. A series of over-expressed and down-regulated genes due to *ERBB2* transformation was identified (Beckers et al., 2005).

Neo-vascularisation and angiogenesis in tumour tissues are critical processes to sustain tumour growth and metastasis. The critical role of the *ERBB* receptor family in angiogenesis and the requirement of *Erbb2* in cardiac development have been previously demonstrated (Lee et al., 1995; Petit et al., 1997; Russell et al., 1999). The inhibition of oncogene mediated angiogenesis contributes to the tumoricidal effect of therapeutics used in the treatment of ERBB2 over-expressing breast carcinomas (Izumi et al., 2002; Klos et al., 2003). Correspondingly, we found that in *ERBB2* transfected fibroblasts several key

factors of angiogenesis were differentially regulated. For example, *Serpinf1*, the gene coding for the pigment epithelium-derived factor (PEDF), was repressed in *ERBB2* transformed cells. It was previously identified as a major inhibitor of vascularisation in prostate and pancreas of *Serpinf1* loss-of-function mice (Dawson et al., 1999; Doll et al., 2003). Also, exogenous PEDF treatment suppressed xenograft tumour growth and induced endothelial apoptosis (Doll et al., 2003). Similarly, other anti-angiogenic factors such as the secreted inhibitors SPARC and TIMP3, were strongly down-regulated in the *ERBB2* transfected cells analysed here (Chlenski et al., 2002; Qi et al., 2003). In contrast, we observed increased expression of the intestinal-enriched Krüppel-like zinc-finger transcription factor *Klf5* (*Bteb2, Iklf*) in *ERBB2* transfected cells. Mice heterozygous for a *Klf5* knock-out allele show impaired angiogenic activity consistent with KLF5 expression in activated endothelial cells (Shindo et al., 2002). Furthermore, *Klf5* was also identified as one of the factors induced upon vascular injury (Kruse et al., 2004). Transfection of *Klf5* in NIH3T3 fibroblasts positively regulated anchorage-independent proliferation and led to loss of cell-cell contact inhibition (Sun et al., 2001). Thus, the observed *Klf5* over-expression may contribute at least partially to the transformed phenotype of *ERBB2* transfected cells. It is also noteworthy that *Tnfaip2* (induced in *ERBB2* transfected cells) was originally identified as a *Tnfa*-inducible response gene that was over-expressed in an *in vitro* model of angiogenesis (Sarma et al., 1992). Similarly, *Sema3c* induced in *ERBB2* expressing fibroblast cells was recently associated with vascular development and angiogenesis in endothelial cells of adult lung (Favre et al., 2003). Taken together these observations support previous findings that one of the biological processes triggered by *ERBB2* over-expression is the induction of angiogenesis. Genes associated with angiogenesis were recently identified as being regulated upon treatment with trastuzumab in ERBB2 overexpressing human breast tumour cells (Izumi et al., 2002). We identified additional angiogenic factors that may serve as further targets to limit growth in ERBB2 over-expressing tumours.

In our expression-profiling analysis we also found a significant fraction of differentially expressed genes that is functionally associated with cell and cell-matrix adhesion (Beckers et al., 2005). This includes up- as well as down-regulated genes suggesting that over-expression of the *ERBB2* oncogene may

affect the balance of factors required also for the adhesive system. This observation corroborates findings of other gene expression-profiling studies in *ERBB2* overexpressing human breast cancer cell lines and breast tumours (Bertucci et al., 2004; Wilson et al., 2002). In addition, we find that many genes regulated in NIH3T3 transfected cells, such as *Ndr1*, *Fn1*, *Sparc*, *Igfbp* and others were also differentially expressed with the same tendency in *ERBB2* transfected human mammary luminal epithelial cells (Mackay et al., 2003). This also supports the relevance of the in vitro model used in our analysis.

As evident from the two examples on the regulation of angiogenic and cell adhesion genes, the assessment of differentially expressed genes with known functions is useful to monitor biological processes that are triggered in expression-profiling experiments. In addition, gene chip expression-profiling of *ERBB2* over-expressing cells identified at least 31 regulated genes that were, so far, not annotated with a predicted biological process or molecular function. Expression-profiling per se is not a tool for the functional annotation of genes. However, the finding that not yet annotated genes are regulated puts them in the functional context of Erbb2. This provides a basis for further experimental studies that then provide more direct information on gene function (Beckers et al., 2002; Nadeau et al., 2001).

RNA expression profiles of prion infected mouse neuronal cells

Prion diseases are a class of transmissible neurodegenerative diseases that include Creutzfeldt-Jakob disease (CJD) in humans, scrapie in sheep and goats and bovine spongiform encephalopathies (Aguzzi and Polymenidou, 2004; Collinge, 2001; Lasmezas, 2003; Prusiner, 1998; Weissmann, 1995). A key event in prion diseases is the accumulation of an abnormal isoform (PrPSc) of a host-encoded protein, termed prion protein (PrPc), predominantly in the nervous system of the infected host (Aguzzi and Heppner, 2000; Nunziante et al., 2003; Prusiner, 1991). There is increasing evidence that PrPSc is the sole component of the infectious agent (Legname et al., 2004). The distinct pathology in prion disease is neuronal loss and astrogliosis, with the latter potentially a bystander effect. For neurological diseases such as Alzheimer's disease (AD), Sandhoff's or Tay Sachs disease, large scale studies of differential gene expression have been performed (Colangelo et al., 2002; Doyu et al., 2001; Myerowitz et al., 2002). Expression-profiling of scrapie and CJD infected material have either

focused on small subsets of genes or have used mixed cell types in whole brain studies (Baker and Manuelidis, 2003; Doh-ura et al., 1995; Riemer et al., 2004; Riemer et al., 2000; Xiang et al., 2004). Given the diverse cell composition of the nervous system, it is likely that the differential regulation of many genes will be obscured in mixed cell or whole brain studies. For example, the expression profiles of purified human microglia infected with CJD revealed a subset of genes involved in processes such as inflammation, cytoskeleton organization, and signal transduction which would likely be obscured or more difficult to interpret from unpurified brain material (Baker and Manuelidis, 2003). The changes in the neuronal gene expression following infection with prions had not been extensively characterized.

The determination of the effects of prion infection on gene expression levels in neuronal cells was the aim of our study. We used scrapie-infected Neuroblastoma cells (N2a) (Butler et al., 1988) and hypothalamic neuronal cells (GT1) (Schatzl et al., 1997). Although, N2a cells are extensively used to characterize prion biogenesis, the cells themselves do not exhibit pathological changes associated with infection in vivo. In contrast, GT1 cells exhibit both apoptosis and vacuolarization reminiscent of the responses observed in the CNS of animals dying from prion disease (Schatzl et al., 1997).

Using our close to genome wide DNA-chip for gene expression-profiling, we have identified several hundred differentially expressed genes in the two cell lines when infected with prion strain RML (Greenwood et al., 2005). ScN2a and ScGT1 cells demonstrate unique changes in RNA profiles and both differ from the reported changes in human microglia and prioninfected brain studies albeit with some overlap. In addition, several of the identified changes are shared with other neurodegenerative diseases such as Alzheimer's disease. The results illustrate that prion infection differs in effect depending on cell type, which could be exploited for diagnostic or therapeutic intervention.

Perspective

Our ongoing projects include the continuation of national and international collaborations to perform expression-profiling experiments on cell culture samples as well as on tissues of mouse models. Several of these collaborations are at the stage of submitted manuscripts, but are not yet included in this

manuscript. Here, a brief overlook of novel technologies that may further improve microarray-based expression-profiling will be attempted. Also, we have provided a proof-of-principle that molecular phenotyping is suitable for studying the effect of environmental factors on gene expression in the mammalian genome. Genome and environmental interactions will be an additional focus of coming projects in the German Mouse Clinic. Therefore, our findings from the pilot study will be briefly summarized.

3.1.8. Rising technologies in the microarray field.

We are making every effort to keep our gene expression-profiling platform at the highest quality standards (Figure 3A, B). As example, we have recently added several hundred probes to our custom made microarray that were previously missing in the Lion mouse arrayTAG clone set. These include genes that are particularly relevant for developmental and neurogenic processes, since several of our projects have this topic as subject. Also, we have improved the chip-to-chip reproducibility by the automation of hybridisation, washing and drying. Recently improved protocols for the amplification of cDNA have allowed us reducing the amount of total RNA from 15µg to less than 500ng used for our dual-colour custom microarray hybridisations (Figure 3C, D). In parallel, we have established two commercial microarray platforms that have superior densities and chip quality, the Affymetrix GeneChip and the Illumina expression beadchip systems.

Significant improvements in the field of microarrays may come from emerging applications of nano-biotechnology (Fortina et al., 2005). One such nano-device with potential for future applications in gene expression studies may be mentioned: Quantum dots (QDs), colloidal semiconductor nanocrystals with a CdSe core and ZnS shell, have exciting physical properties that may eventually favour them over fluorophore cyanine tags most frequently used in expression profiling experiments today. The absorption spectra of QDs are very broad and their emission is confined to a narrow, symmetrical band that is characteristic of the nano-particle size. They are highly luminescent and stable against photobleaching. DNA-hybridization studies have proven their applicability to the detection of nucleic acids (Byers et al., 2007; Han et al., 2001). QDs can be fabricated for any emission spectrum and excited at a single wavelength. Furthermore, defined quantities and ratios of different colour QDs have already

been packed into microbeads and subsequently linked to distinct nucleic acid probes. The relative ratios of colours label the identity of the probe. Such microbeads with three different colour QDs have been detected at the single bead resolution in DNA hybridisation studies (Han et al., 2001). Coding nucleic acid probes with QD loaded microbeads or with fluorescent microbarcodes has evoked the vision of flow systems with tag-by-tag recognition instead of monitoring hybridization to a probe in a fixed position on a microarray (Dejneka et al., 2003; Gao and Nie, 2005).

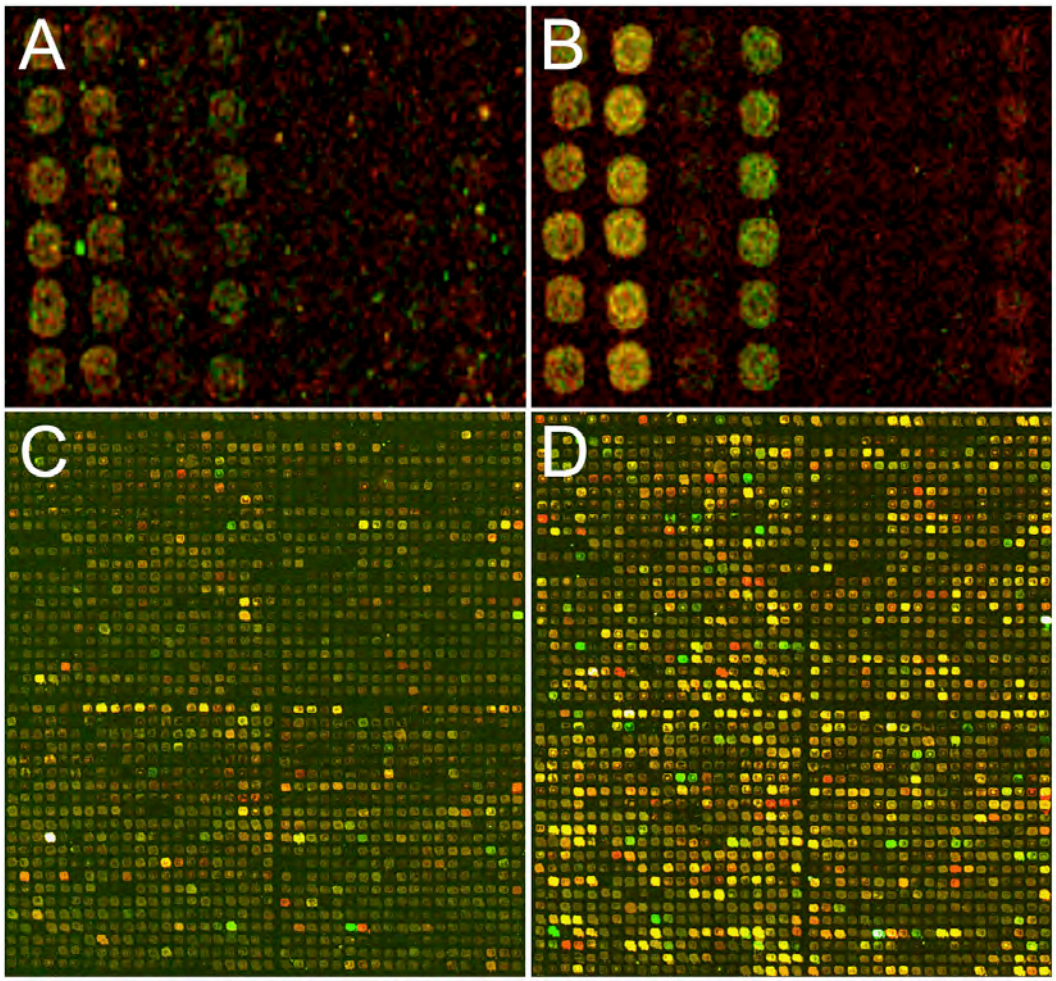

Figure 3: *Technical modifications to ensure high quality microarray data and sensitive detection of gene expression. Hybridisation of identical target RNAs to probes spotted on a Telechem silylated slide (A) and a Telechem SuperAldehyde slide (B). Sensitivity and signal to noise ratio are improved in (B). The microarray in (C) was hybridised with labelled cDNA from 15µg total RNA from liver and kidney. The identical RNA samples were used in (D) except that 500ng were used to produce amplified RNA (TargetAmp™). Signal intensities in (D) are generally higher than in (C).*

Increased specificity and signal intensities are desirable, in particular for the detection of low abundant messages and small RNAs, such as miRNAs, whose importance for gene regulation is starting to be recognized. Locked nucleic acids (LNAs) are nucleic acid analogues that contain one or more LNA nucleotide monomers with a bicyclic furanose unit locked in an RNA mimicking sugar conformation. LNA oligonucleotides display unprecedented hybridization affinities to complementary single-stranded RNA and complementary single- or double-stranded DNA (Vester and Wengel, 2004). Using LNAs as oligonucleotide probes for in situ hybridisation distinct spatial expression patterns have been detected in zebrafish and mouse embryos (Kloosterman et al., 2006) and in formalin-fixed, paraffin-embedded human tissues (Nelson et al., 2006). Subsequently LNA probes have also been used to produce microarrays for the sensitive and specific detection of miRNA expression in total RNA extracts from different mouse organs (Castoldi et al., 2006). Other strategies for the detection of miRNAs in a microarray format have successfully combined the direct labelling of miRNAs with cyanine dye und an optimised probe design that enhances the specific binding to miRNAs (Wang et al., 2007).

3.1.9. Molecular phenotyping of genome-environment interactions.

The conceptual framework of the German Mouse Clinic is currently being extended towards the analysis of phenotypes caused by environmental factors. Five areas of major interest for human health have been identified: physical exercise, infection, pollution of the air, stress and nutrition. As a pilot study we have investigated the effect of creatine supplemented diets on healthy aging in mice (Bender et al., 2007). Mice that were fed on a 1% creatine diet for a year had an extended healthy life-span, reduced levels of several biomarkers for aging and for energy metabolism. Gene expression-profiling of brain tissue identified regulated genes associated with behavior, neurogenesis, energy pathways and protein biosynthesis in mice on creatine supplemented diet (Bender et al., 2007). Many genes regulated in the aging brain were reversely regulated in the brain of mice under creatine-supplemented diet. These data provided a proof-of-principle that the screens of the GMC are generally compatible with the identification of environment related phenotype variations.

3.2. COMPARATIVE TRANSCRIPTOMICS AND PROTEOMICS.

Introduction

3.2.1. From proof-of-principle to neurodegenerative disease models in the Human Brain Proteome Project

Most biochemical processes within and between cells are put into effect by the interaction between proteins, or between proteins and their substrates (Ge et al., 2001; Li et al., 2004; Walhout and Vidal, 2001). The proteome of a cell is the result of controlled biosynthesis, and hence largely (but not exclusively) regulated by gene expression (Kanapin et al., 2003). In turn, gene expression can be regarded as a sensitive read-out of the biochemical state of the cell. Thus, transcriptome and proteome feed back to each other in a highly complex way. To begin to understand the mutual regulatory interactions between transcriptome and proteome, a comparative approach including the simultaneous monitoring of expression at the RNA and protein levels is required.

As a first step towards this goal we have done a comparative analysis of transcripts and proteins in liver and kidney of the mouse (Mijalski et al., 2005). As described in 3.2.2., the analysis suggested concordance between protein and gene expression when these two tissues are compared. In the second step (3.2.3.), we applied the comparative approach to the analysis of a mouse model for renal dysfunction (Frey et al., 2007). The data were complemented by measurements of metabolites, thus adding a third level of regulation. Only through the system-wide identification of components, the quantification of their regulation in the mutant tissue and the integration of the data in a model, it was possible to define the functional role of the *Slc15a2* gene in the kidney.

The methodological progress that has been made in genome sequencing and high-throughput measurements of molecules in the cell has renewed the interest in a system-level understanding of biological processes (Kitano, 2002). A previous report has described the integrative analysis of transcriptome, proteome and metabolites in *E. coli* in response to perturbations (Ishii et al., 2007). A series of 24 viable mutations that disrupt glycolysis or the pentose posphate pathway was analysed. A major observation in the *E. coli* mutants was that global changes of expression and metabolite levels were very low as compared to the changes that occur in response to variations in growth rate. Thus, the investigated metabolic networks in *E. coli* appear to be very robust against

perturbations. The successful integration of the three levels of data evoked the suggestion of a generalised road map towards systems biology (Sauer et al., 2007). Conceptually, many aspects of this road map are included in our study of renal dysfunction in kidneys of the *Slc15a2* mutant mice.

Because a living cell, an organ, or an organism is not just an assembly of genes, transcripts and proteins, its properties cannot be fully understood by the quantitative measurement of the components. As one step further towards a more holistic and functional theory we are currently extending our attempts to perform measurements at the systems level (3.2.4.). In a mouse model for neurodegenerative disorders, we are currently including the topological analysis of co-localisations of proteins at the sub-cellular level in distinct brain regions (Schubert, 2003; Schubert et al., 2006). Since the function of proteins often depends on their presence in specific compartments of the cell or in protein complexes, we hope that we will achieve the addition of this topological information to the systems analysis.

Own research

3.2.2. Comparative analysis of transcriptome and proteome in mouse liver and kidney

To explore the general feasibility of a comprehensive and comparative gene expression analysis we used RNA expression-profiling and 2D-gel electrophoresis combined with mass fingerprinting (MFP) of mouse liver and kidney. The basic technologies for genome wide expression analyses at the mRNA (Lockhart et al., 1996; Pease et al., 1994; Schena et al., 1995) and protein levels (Gauss et al., 1999; Gorg, 1993; Klose et al., 2002; Tsugita et al., 2000) were available at the time of our study. Transcript profiling had been used to assess normal variability in gene expression levels of mouse liver, kidney and testis (Pritchard et al., 2001) and to analyse changes in expression patterns during embryonic and fetal liver development (Jochheim et al., 2003). Comparative transcriptome and proteome analyses in complex organisms were and still are very limited and have been performed in human platelets (McRedmond et al., 2004) and heart tissue (Ruse et al., 2004), and the Anopheles and Culex salivary glands (Ribeiro et al., 2004; Valenzuela et al., 2003). In rodents the proteome of mouse primary islet cells was correlated with

RNA expression data of purified primary rat beta cells. This data suggested a close correlation between mRNA and protein expression (Cardozo et al., 2003). A parallel analysis of transcripts and proteins at a genomic scale in identical mouse tissue samples had not been performed.

Using our custom made microarray with over 20.200 probes, we identified more than 1.800 transcripts differentially regulated with high statistical significance between mouse liver and kidney. 2D-gel electrophoresis detected around 2300 spots in each organ. About 800 spots were regulated with a factor of at least 1.5-fold. PMF of 47 isolated spots resulted in the identification of 43 distinct differential proteins (Mijalski et al., 2005). We used this rather comprehensive gene expression data set as a tool to (i) evaluate functions of differential transcripts and proteins, (ii) relate transcriptional and post-transcriptional regulation, and (iii) map differential transcripts to the mouse genome.

Functions of differentially regulated transcripts and proteins

The comparison of the functional annotation of the major differential proteins and transcripts suggests that protein and transcript detection methods reveal functional categories with different preference. Metabolic enzymes constitute the largest fraction of identified proteins. A minor fraction is associated with other functions such as transport or structure. These observations corroborate similar findings made, for example, in the analysis of the mouse brain proteome (Gauss et al., 1999; Tsugita et al., 2000). In contrast, differential transcripts have more diverse functions (Mijalski et al., 2005). On one hand, the relatively low number of diverse functional groups at the protein level may be due to current limitations of the proteome analysis method. We estimated the detection limit of the proteomics approach to at least 1000 copies of a protein per cell. The proteins detected by 2D-gel electrophoresis represent only the fraction of the most abundant proteins. In addition, we selected the most differential spots for protein identification. This experimental limitation is probably one important reason why the detected proteins mostly have metabolic functions. Thus, regarding differences in protein expression a major distinction between liver and kidney cells appears to be the set of metabolic enzymes activated in the respective tissue. The better sensitivity of DNA-chip expression profiling may be one reason why the differential transcripts have more diverse functions. The transcriptome data included 22% (liver) and 36% (kidney) novel genes and genes without

functional annotation (Mijalski et al., 2005). Thus DNA-chip based transcriptome analysis may also be an efficient method for the identification of novel disease associated genes (Dhanasekaran et al., 2001; Su et al., 2001).

Comparing transcriptome and proteome of kidney and liver

The comparative approach opened the possibility to relate regulation at the transcript and post-transcriptional levels. In our experimental set-up we can easily analyse the expression at the transcript level of differentially expressed proteins since all probes on our DNA-chip have been sequenced. The reverse, finding the corresponding protein for a differential transcript on the 2D-gel, would require specific antibodies or a systematic MFP analysis of all spots on 2D-gels. The majority of the differential proteins was also regulated with the same tendency in DNA-chip analyses (Mijalski et al., 2005). This observation suggests that at least for the most differential proteins gene expression at the transcript level correlates well with protein expression. Similarly, a close correlation between mRNA and protein expression was suggested in rodent pancreatic islets cells (Cardozo et al., 2003) and mitochondria from distinct mouse tissues (Mootha et al., 2003). Five differential proteins (Rnf20, Actr3, Mtx2, Ak1, and Dnahc11) were not regulated at the RNA level (Mijalski et al., 2005), suggesting that the differential expression of these proteins could be due to the stability or differences in secretion or accumulation of these proteins. Moreover, Fh1 and Fah, both abundant proteins in kidney, were strongly transcribed in liver but not in kidney possibly suggesting different turnover rates or efficiencies of translation in the two tissues. The comparison of gene regulation at the transcript and protein levels thus provides a proof-of-principle for the usefulness of the comparative approach.

Co-expressed genes co-localize in the genome

Our transcriptome analysis of two functionally distinct tissues led to the identification of more than 1000 differentially expressed genes. This allowed the assessment of chromosomal co-localizations. We found 29 genomic clusters of co-expressed genes (Mijalski et al., 2005). Chromosomal regions of co-expressed genes have also been identified based on expression profiling data in yeast, C. elegans, Drosophila, man and mouse (Caron et al., 2001; Cohen et al., 2000; Roy et al., 2002; Spellman and Rubin, 2002; Su et al., 2004). The co-

regulation of closely linked genes through shared sequence elements in *cis* (such as enhancers, repressors, insulators, LCRs, MARs, SARs etc.) has been described for gene families such as *apoE*, *α-globin*, *β-globin*, *Hox* genes and others (Allan et al., 1997; Engel and Tanimoto, 2000; Li et al., 1999; Spitz et al., 2003). Our expression data identified the proximal *Serpin* subcluster as linked and differentially regulated genes (Mijalski et al., 2005). The arrangement of these genes is conserved between mouse and man except that the human *SERPINA1* gene has five isoforms in mouse (Goodwin et al., 1997). Recently, a control region was identified in the human locus that is required for *SERPIN* gene activation and for chromatin remodelling of the proximal subcluster (Marsden and Fournier, 2003).

The co-regulation of linked genes may be imposed either by sharing *cis*-regulatory interactions or, alternatively, may be associated with a more general or long-range property of genomic sequences (Li et al., 2002; Spitz et al., 2003; Zakany et al., 2004). Additional data suggest that at least some of the genes, identified in our work as co-expressed (Mijalski et al., 2005), may indeed be co-regulated through the same regulatory factors. For example, the expression of the *Serpin* and the *Fetuin* clusters in liver may at least in part require the same transcription factors. Human *HNF3* (*Foxa3* in mouse) is an essential factor for the transcriptional regulation of many hepatic genes that can affect chromatin structure by displacing linker histones at least in the serum albumin enhancer. It was also suggested to be one of the potential factors regulating expression of *SERPIN* genes (Cirillo and Zaret, 1999; Costa et al., 1989; Marsden and Fournier, 2003). HNF3 binding sites were also identified in the liver specific *FETUIN* (*AHSG*) gene. In the mouse, *Ahsg* and *Fetub* are direct neighbouring genes within approximately 50kb on chromosome 16 (Chr. 3 in man) (Denecke et al., 2003). Both genes, *Ahsg* (two probes, rank #9 and #11), and *Fetub* (rank #636), were strongly expressed in liver and weakly expressed in kidney. Based on these observations we hypothesize that the co-localization of co-expressed genes in our study may at least in part be of functional relevance.

3.2.3. Combining transcriptomics, proteomics and metabolomics in the *Pept2* mutant analysis.

One step towards systems biology.

In the second comparative project we set out to elucidate the role of Slc15a2 (Pept2) in renal amino acid metabolism. We submitted kidney tissues of wild-type and *Slc15a2*$^{tm1Dan/tm1Dan}$ mouse lines to a combined transcriptome, proteome and metabolome profiling and analysed urinary amino acids and dipeptides. Slc15a2 is an integral membrane protein in the apical membrane of renal epithelial cells that operates as a rheogenic transporter for di- and tripeptides and structurally related drugs (Rubio-Aliaga et al., 2000). Unexpectedly, kidneys of mice that lack the Slc15a2 transporter do not show prominent phenotypic alterations (Rubio-Aliaga et al., 2003). However, the combined DNA-microarray, proteome and metabolite analyses detected numerous subtle, yet significant changes (Figure 4). The data suggested that Slc15a2 is predominantly a transporter for re-absorption of cys-gly originating from glutathione (GSH) break-down (Frey et al., 2007). Although each profiling technique used in this study has its limitation in terms of sensitivity or interpretation of biological relevance, their combination for the first time allowed to identify alterations in renal handling of peptides, amino acids and derivatives in these Slc15a2 transporter-deficient animals (Figure 5).

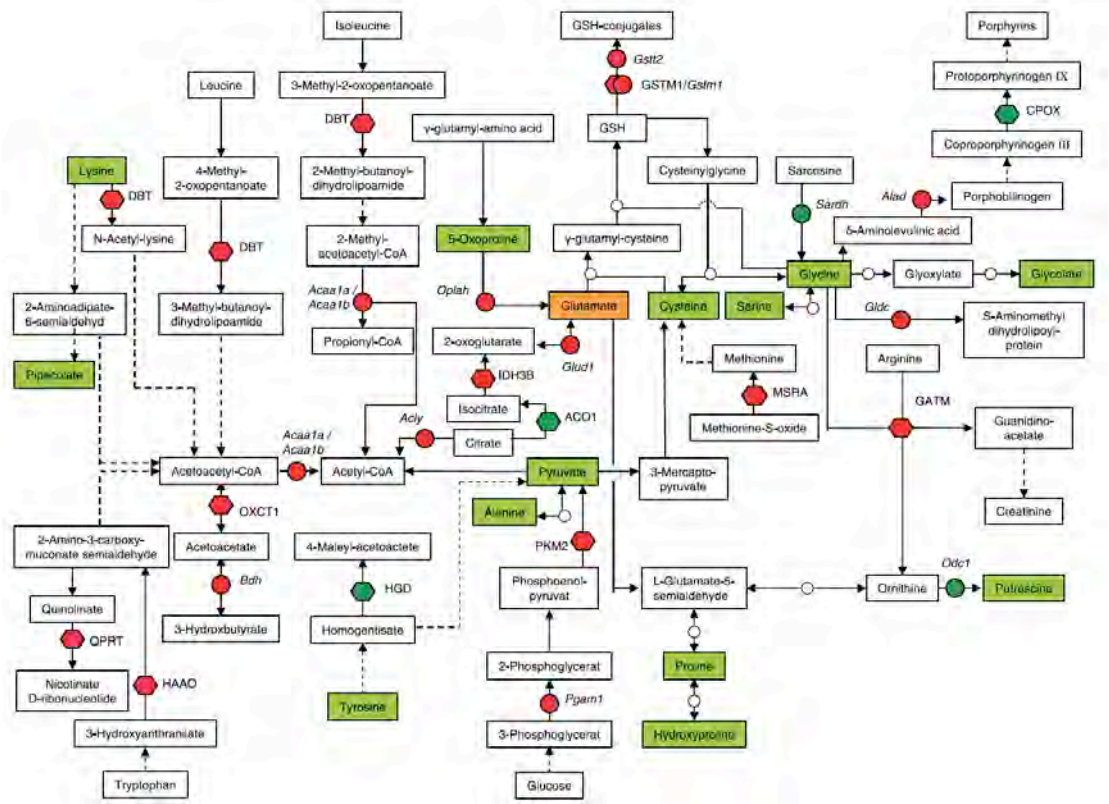

Figure 4: *Combined analysis of transcriptome, proteome and metabolites in kidneys from Slc15a2$^{tm1Dan/tm1Dan}$ mutant mice (Frey et al., 2007). Metabolites are shown in boxes, green boxes indicate reduced levels, and orange boxes indicate increased levels of metabolites in mutant kidneys. Red and green circles indicate up- and down-regulated genes, and red and green hexagons indicate up- and down-regulated proteins in mutant kidneys.*

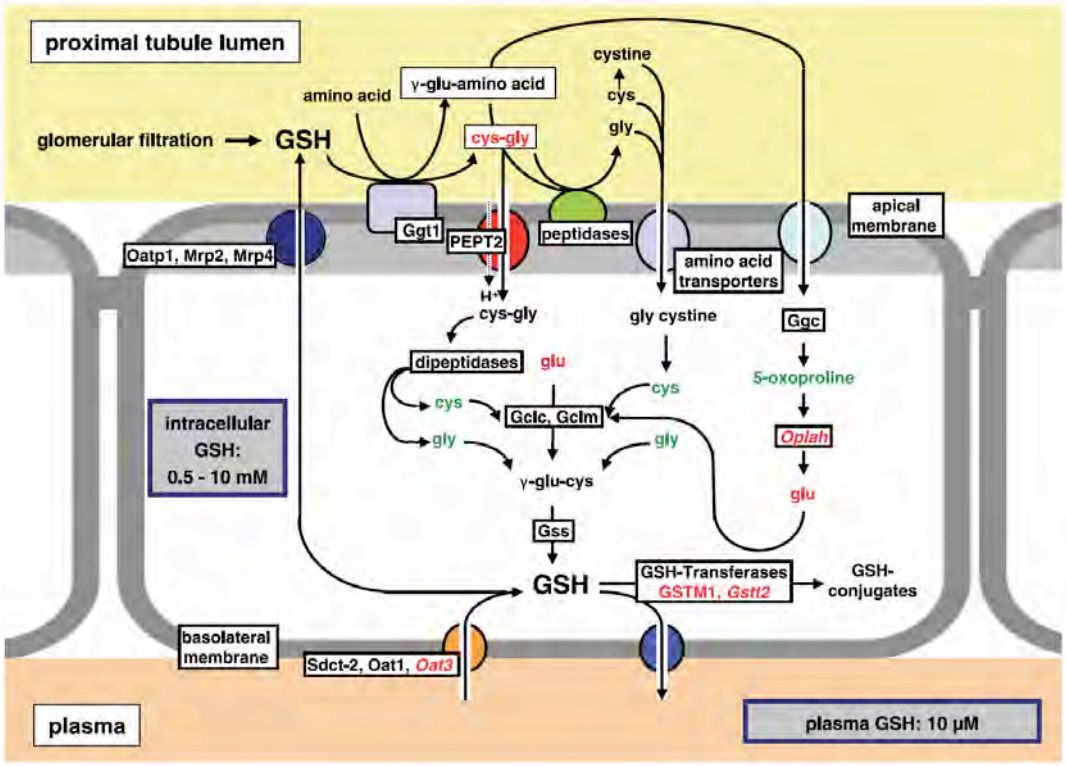

Figure 5: *Scheme of GSH metabolism in epithelial cells of the proximal tubule (Frey et al., 2007). GSH is degraded and resynthesized in proximal tubular cells via the gamma-glutamyl-cycle. By reabsorption of cys-gly, Slc15a2 (Pept2) could deliver substrates for re-synthesis of GSH. Enzymes, transporters, and metabolites with increased expression or concentration in the transporter deficient animals are labeled in red, and entities with decreased expression or concentration are labeled in green.*

Perspectives

3.2.4. A systems approach towards a better understanding of a mouse model for ageing related, neurodegenerative diseases.

As a central project of the Human Brain Proteome Project (HBPP2), we are currently combining transcriptomics and proteomics together with a focused pathological examination of brain tissues and the complete GMC phenotyping screen for the comprehensive analysis of a mouse model for Alzheimer's disease. In addition, we also include an analysis of the subcellular co-localisation and anti-colocation of proteins at the subcellular resolution in specific brain regions. This has become possible through the so called multi-epitope-ligand cartography (MELC) which can generate a toponome map of up to a hundred proteins in one tissue sample using sequential rounds of fluorescent in situ detection (Schubert, 2003; Schubert et al., 2006). This allows the identification of changes in the distribution of proteins (and hence their interaction and function) that may not be detected by standard proteomics methods. As model for Alzheimer's Disease we have selected the UBB+1 transgenic mouse line. This mouse models recapitulates the expression of an aberrant form of *Ubiquitin-B* that is also found in neuronal intracellular protein inclusions caused by transcriptional misreading (van Leeuwen et al., 1998). High levels of mutant UBB+1 protein lead to an impairment of the ubiquitin-proteasome system (UPS) which is a suspected cause of neurodegeneration (Hope et al., 2003; van Tijn et al., 2007). The preliminary data from the GMC and our gene expression profiling analyses of cortex and hippocampus biopsies suggest that the UBB+1 transgenic mice are characterised by subtle behavioural and gene expression changes. The combination of the different phenotype levels in our analysis of young and old transgenic mice will hopefully support the understanding of the etiology of tauopathies that are characterised by intracellular UBB+1 accumulation.

3.2.5. Which is better: Transcriptomics or proteomics?

There is a continuous controversy concerning the question which of the post-genomic research approaches, transcriptomics or proteomics, is the more informative, interesting and rewarding research topic. The investigation of the full complement of transcripts has become a robust method. It can be performed at a high-throughput rate and it is cost-effective. Tens of thousands of mRNAs can be measured quantitatively in a single experiment and some recent microarray

platforms are specifically designed to differentiate alternative splice products, which have been estimated to exist for more than half of the mammalian genes (IHGSC, 2001; Waterston et al., 2002). In contrast, the proteome analysis is still more limited in breadth and depth of coverage. This is mostly due to the higher number, higher variability in abundance, and higher biochemical diversity of proteins as compared with transcripts. This natural premiss demands more sophisticated, sensitive and specific methods for the faithful portray of the full protein complement. Still, an increasing number of studies - including our own ones - has compared and contrasted transcriptomics and proteomics data (Chen et al., 2002; Griffin et al., 2002; Ideker et al., 2001; Washburn et al., 2003). Concordance of regulation between proteins and transcripts in these studies ranged from a maximum of 60% to a complete lack of correlation. In addition to several potential biological reasons (such as alternative splicing, post-transcriptional and post-translational modifications, differences in the turn-over of mRNAs and proteins, distinction between mRNA in monosomes or polysomes and so on), technical issues may contribute to the imperfect correlation between transcriptomic and proteomic data (Hegde et al., 2003). Independent of the origin and degree of correlation, there is no doubt that transcriptome and proteome are not identical. Transcriptomic and proteomic approaches each provide unique perspectives and their analyses complement each other. As briefly pointed out above, transcriptomics is particularly capable of high-throughput, genome-wide examinations. Accordingly there has been and there is the great expectation that this type of analysis may result in a better understanding of the molecular mechanisms underlying disease. It is hoped that this understanding is fundamental to the rational development of targeted and individualised therapies. Transcriptomics has been highly successful in the identification of large numbers of potential biomarkers and targets in oncology (see 3.1.1.). One important reason, why it is the field of oncology where transcriptomics is thriving, is the fact that tumour biopsies are relatively easy to collect from patients. Secreted proteins and transmembrane proteins are particularly desired targets for therapy, since pharmaceutical compounds may easily interact with them and modulate their function without entering the cell. One strategy that has been successfully applied is the combination of a transcriptomic screening approach with a well-directed assessment of a limited number of proteins as potential candidates for

diagnostic, prognostic or therapeutic purposes (Kim et al., 2002; Miller et al., 2003; Mok et al., 2001; Shvartsman et al., 2003; Tanwar et al., 2002; Welsh et al., 2003; Zhou et al., 1998). Other human samples that can be used for transcriptomics and proteomics, and which are relatively easy to collect are blood and to some extend cerebrospinal fluid. Despite the enormous number of candidate target genes that have been identified from tumour tissues and blood samples in the last decade, only a very limited number has made its way to clinical application. Given the inherent limitations of both approaches, the combination of transcriptomics and proteomics appears to be the most rewarding strategy not only for progression of pharmaceutical applications, but also for the basic understanding of the molecular biology that underlies diseases. Gleevec and Herceptin are two commercial cancer drugs (Drevs et al., 2003), which specifically target cell-surface proteins, and which are prime examples for the described combination of global transcriptomics and targeted proteomics.

3.3. DELTA/NOTCH SIGNALLING FUNCTION DURING MOUSE EMBRYOGENESIS.

Introduction

3.3.1. From novel *Dll1* functions to new target genes and back to gene functional studies.

A large number of recent reviews has dealt with Delta/Notch gene functions in pleiotropic biological processes and their evolutionary conservation in different species (Cheng and Kopan, 2005; Efstratiadis et al., 2007; Grabher et al., 2006; Rida et al., 2004; Saga, 2007; Shi and Stanley, 2006). A topical review has concisely summarized the historical isolation of notched wing mutants in *Drosophila* and the subsequent discovery of the Delta/Notch gene family also in mammals. It gives short summaries of the diverse Delta/Notch signalling functions in cell fate determination and patterning during embryogenesis, in the central nervous system, in haematopoietic stem cells, in melanoblast survival, in vasculature formation, during organogenesis, and post-developmental stem cell maintenance, for example, in intestinal epithelia and for muscle regeneration (Chiba, 2006).

Our own research has centred on the mouse *Delta-like 1* (*Dll1*, *Delta1*) gene and its function predominantly during somitogenesis. We have identified a novel functional requirement of *Dll1* for the determination of the left/right asymmetry and the involvement of *Dll1* in the development of intervertebral joints and discs. The canonical Delta/Notch model of lateral inhibition and cell-cell signalling adequately explains only a few of the pleiotropic developmental processes. We, therefore, hypothesized that a large number of factors associated with Delta/Notch signalling have not yet been identified. Several of our projects focused on the identification of novel *Dll1* target genes, direct interactors of *Dll1* and novel mutant alleles that interact genetically with a *Dll1* loss-of-function mutation. Some of the identified genes are now object of ongoing gene targeting and functional studies.

Own research

The studies summarized below, were done in collaboration with the group of Martin Hrabé de Angelis, Institute of Experimental Genetics, GSF. For the projects described in 3.3.2 to 3.3.4 the largest part of the work was done in his

laboratory, whereas the projects under 3.3.5. and the work under 3.3.6. were dominated by contributions of my laboratory.

3.3.2. The earliest function of the mouse *Dll1* gene.

Determination of left/right asymmetry.

Apart from screening for novel components of Delta/Notch signalling, our work has also contributed to the functional annotation of the *Dll1* gene itself. As an example, we reported that Notch signalling is required also for normal left-right determination in mice. We showed that the loss-of-function of the *Dll1* gene (*Dll1*$^{tm1Gos/tm1Gos}$) causes a situs ambiguous phenotype, including randomisation of the direction of heart looping and embryonic turning. The most probable cause for this left-right defect in the *Dll1* mutant embryos was a failure in the development of proper midline structures. These originate from the embryonic node, which is disrupted and deformed in *Dll1* mutant embryos. Based on expression analyses in wild-type and mutant embryos, we suggested a model, in which Notch signalling may be required for the proper differentiation of node cells and node morphology (Przemeck et al., 2003). This is the earliest function that has been attributed to the *Dll1* gene, so far.

3.3.3. Identification of *Magi2* as intracellular interactor of *Dll1*.

Watch out: Oncoming traffic!

We identified Magi2 as a factor that interacts with the intracellular domain of the Dll1 gene product by means of in vitro pulldown assay and in a mammalian two-hybrid system in vivo (Pfister et al., 2003). Taking into consideration the described function of MAGUK proteins in the assembly of intracellular multiprotein signalling complexes and the existence of a nuclear localization signal in the Dll1 intracellular domain, these findings suggested that the interaction of Dll1 and Magi2 might trigger an intrinsic signal in the Dll1 expressing cell. This is an important finding considering that in the canonical Delta/Notch signalling model Delta is generally regarded as the ligand, and Notch is regarded as the receptor.

3.3.4 A phenotype based ENU mutagenesis screen for *Dll1* modifiers. A model-screen for complex phenotypes.

To identify novel alleles that synergistically interact with the *Dll1* loss-of-function allele (*Dll1*$^{tm1Gos/tm1Gos}$), we carried out a phenotype-driven modifier screen.

Heterozygous Delta1 knockout animals were crossed with ENU mutagenized mice. The offspring were screened for dysmorphologies, and clinical chemical and immunological variations that require one copy of the *Dll1* loss-of-function allele. First, we could show that heterozygous *Dll1* mutant mice have reduced body weight and distinctly altered clinical chemical parameters. Subsequently, we isolated 35 new mouse mutant lines in the sensitized mutagenesis screen based on changed phenotypes. Of major interest are seven mutant lines that exhibit a *Dll1*-dependent phenotype (Rubio-Aliaga et al., 2007). These mutant mouse lines provide excellent in vivo tools to study the role of Notch signalling in kidney and liver function, cholesterol and iron metabolism, during cell-fate decisions and maturation of T cells in the immune system.

Many human diseases with adult onset show familial accumulation that does not follow Mendelian inheritance. Such complex diseases are conditions that are influenced by interactions of multiple alleles. Combining the gene targeting technology and ENU mutagenesis in mice, we have provided the demonstration that modifier of phenotypes can efficiently be isolated in this set-up.

3.3.5. Identification of *Dll1* target genes using differential expression-profiling

The Delta/Notch signalling pathway is required for cell differentiation and patterning processes during mammalian embryogenesis including lateral specification during neurogenesis (Beatus and Lendahl, 1998; Morrison et al., 2000; Yun et al., 2002), determination of left and right body halves (Krebs et al., 2003; Przemeck et al., 2003; Raya et al., 2004), patterning of the presomitic mesoderm (Dale et al., 2003; Jouve et al., 2000; Takahashi et al., 2003) and establishment and maintenance of somite boundaries (Barrantes et al., 1999; Hrabe de Angelis et al., 1997). The classical model of Notch signalling cannot explain these pleiotropic gene functions and developmental processes satisfactorily. Cross-regulation exists, for example, between the Notch and Wnt pathways during patterning of the presomitic mesoderm (Aulehla et al., 2003; Hofmann et al., 2004). The *Dll1* (*delta-like 1, Delta1*) gene encodes one of at least five known ligands of Notch receptors in mice (Bettenhausen and Gossler, 1995; Bettenhausen et al., 1995). To identify novel Notch signalling targets on the RNA and protein levels, we screened the differential transcriptome and proteome of *Dll1*-deficient ($Dll1^{tm1Gos}$) and wild-type (wt) embryos at day 10.5 post

coitum (E10.5, Theiler stage 17 to 18). Expression of candidate genes was validated by *in situ* hybridization of wt and *Dll1*, *Dll3* and *Jag1* mutant embryos or Western blot analyses, respectively (Machka et al., 2005).

The differential expression-profiling approach identified 47 regulated transcripts and 40 differentially expressed proteins. Only few of these targets, such as *Nes* and the proteasome subunits, were previously identified as regulated by Notch signalling (Mellodew et al., 2004). One reason for the lack of other *Notch* target genes among the top regulated genes is the limitation of the sensitivity of the experimental approach. Genes such as *Mesp2*, *Cer1*, *Uncx4.1*, *Lfng*, *Hes1*, *Hes5*, or *Dll3*, which are regulated in $Dll1^{tm1Gos}$ mutant embryos, were either detected with very low intensities, resulting in unreliable or incomplete data, or were not represented on the microarrays (Barrantes et al., 1999; Jouve et al., 2000). In addition, some of these genes have highly restricted domains of expression at E10.5. However, it is also conceivable that the currently known targets of Notch signalling are not necessarily the most differentially expressed genes.

We used the DNA-chip expression-profiling technique as screening method for unknown direct or indirect *Dll1* target genes and showed that a subset of these genes has restricted expression patterns during development in neuronal tissues, and the somitic and presomitic paraxial mesoderm. In situ hybridizations confirmed altered expression patterns in *Dll1* mutant embryos for the majority of genes. Expression of some of these genes is also affected in *Jag1* and *Dll3* mutants. For example, the reduced expression of *Sema5b* in the neural tube was common to embryos of the $Dll1^{tm1Gos/tm1Gos}$, $Dll3^{pu/+}$, and $Dll3^{pu/pu}$ genotypes (Figure 2 in (Machka et al., 2005)). In contrast, expression of *Csk* was much less affected in homozygous *Dll3* mutants as compared to homozygous *Dll1* mutants.

Similarly, protein expression-profiling identified a set of regulated proteins that may represent novel direct or indirect targets of the *Dll1* signal. The regulated expression of some of these proteins in *Dll1* mutant embryos was confirmed by immuno-Western blot analyses. It is also remarkable that among the down-regulated proteins six are proteasome subunits (*Psma2*, *Psma4*, *Psmb2*, *Psmb4*, *Psmc6*, and *Psmd8*; Supplemental Table in (Machka et al., 2005)).

With the exception of *Atp5h*, none of the differentially expressed genes was identified on both the RNA and protein levels. The down-regulation of *Atp5h* at

the transcript level was also confirmed by reverse transcription PCR. An antibody for use with Western blot analysis was not available. The lack of more extensive concordance of regulation at the RNA and protein levels may be due to current limitations in the sensitivity of the proteomics and transcriptomics approaches. Since both approaches are not exhaustive (detecting only a fraction of the regulated genes) it is rather unlikely to reveal extensive correspondence between transcriptional and translational regulation. In addition, differences in the transcriptional and translational regulation of gene expression may also contribute to our observation of limited concordance. In either case the combination of transcriptomics and proteomics approaches provides a better appreciation of changes in gene expression patterns. The evaluation of the significance of the identified genes and proteins in the context of Notch signalling requires further functional studies.

3.3.6. Compartmentalised expression of *Delta-like 1* in epithelial somites is required for the formation of intervertebral joints

Background

Segmentation along the rostro-caudal (R/C) axis is a fundamental characteristic of vertebrates. It originates during embryogenesis when the paraxial mesoderm is divided bilaterally into spheres of epithelial somites, which enclose a core of mesenchymal cells, the somitocoele cells (Dubrulle and Pourquie, 2004). Each somite is divided in a rostral and caudal compartment with distinct gene expression and developmental fate (Gossler and Hrabe de Angelis, 1998; Gridley, 2006; Pourquie, 2003). This R/C somite polarity is established early on in the presomitic mesoderm (PSM) prior to segmentation (Bronner-Fraser and Stern, 1991; Morimoto et al., 2007; Saga, 2007; Stern and Keynes, 1987; Tam et al., 2000). It is essential for the subsequent resegmentation of sclerotomes (Bagnall, 1992; Bagnall et al., 1988) and the sequential patterning of the neural tube (Keynes and Stern, 1984; Keynes and Stern, 1988).

Experimental evidence suggests that Mesp2 and Notch signalling are required for the initiation of R/C somite compartmentalisation in nascent somites through induction and suppression of *Dll1* expression in caudal and rostral somite halves, respectively (Sato et al., 2002; Takahashi et al., 2003; Takahashi et al., 2000). The maintenance of somite R/C polarity requires the compartmentalised

expression of caudal genes, such as *Dll1*, *Notch1*, *Paraxis* and *Uncx4.1* (Rawls et al., 2000).

We have analysed the requirement of restricted *Dll1* expression in epithelial somites for the maintenance of R/C identity following the initial establishment and for the development of somitocoele cells, in particular. It has been demonstrated that somitocoele cells contribute to proximal ribs, the articular surface of intervertebral (zygapophyseal) joints, and the peripheral parts of the intervertebral discs (IVDs) (Huang et al., 1996; Huang et al., 1994). In the avian embryo, these cells constitute a joint forming compartment, the arthrotome (Christ et al., 2004; Mittapalli et al., 2005). The molecular mechanisms underlying the specification of the arthrotome compartment have not been studied. Our data demonstrate that the over-expression of *Dll1* throughout epithelial somites of transgenic mice does not alter somite polarity but affects the development of intervertebral joints, IVDs and proximal ribs. This suggests a role of restricted *Dll1* expression in caudal epithelial somites for arthrotome development.

Generation of Dll1 gain-of-function transgenic lines

To direct the expression of Dll1 throughout somites including rostral somite compartments, a full length Dll1 cDNA under the control of the mesodermal specific cis-regulatory element (msd) was fused to the *Dll1* minimal promoter (Beckers et al., 2000) (Figure 6A). Two independent and stable transgenic lines (Tg(msd/Dll1)1Ieg and Tg(msd/Dll1)2Ieg) were established by pronuclear injection. Transgenic mice showed tails with multiple kinks and a reduction in tail and body length with varying severity (Figure 6B, C). Both transgenic lines exhibited comparable dysmorphologies of the axial skeleton with varying severity. 25% of the transgenic mice born had an externally visible alteration of the phenotype. 18 transgenic animals without externally visible transgenic phenotypes from both the transgenic lines were examined for morphological changes of the axial skeleton by X-ray imaging (Table 4). Abnormal bony fusions of vertebral bodies were found in the thoracic, lumbar and tail regions in 9 out of the 18 transgenic animals. Vertebrae with reduced rostro-caudal length in the thoracic or tail regions were evident in 7 out of the 18 transgenic mice without externally visible phenotype. In 6 out of the 18 animals the number of thoracic and lumbar vertebrae was altered as compared to wild-type littermates and in 1 out of the 18 animals the number of sacral vertebrae was changed. Neural

arches were irregular (lacking the spinous process or without bony fusion in the midline) in 11 out of the 18 transgenic mice without externally visible phenotype changes. We did not find dysmorphologies of the vertebral column by X-ray imaging in one of the 18 transgenic animals. Bones of limbs and skull were not affected in any of the transgenic animals. The shortening of the vertebral axis in adult transgenic mice with severe transgenic phenotype was due to frequent fusions of vertebral bodies from thoracic to caudal regions and a reduction of the R/C length of vertebrae caudal to the cervical region (Figure 6D, E). Thus, the Dll1 overexpression phenotype was characterized by various dysmorphologies of the axial skeleton (analysed in detail below) with high penetrance but varying severity.

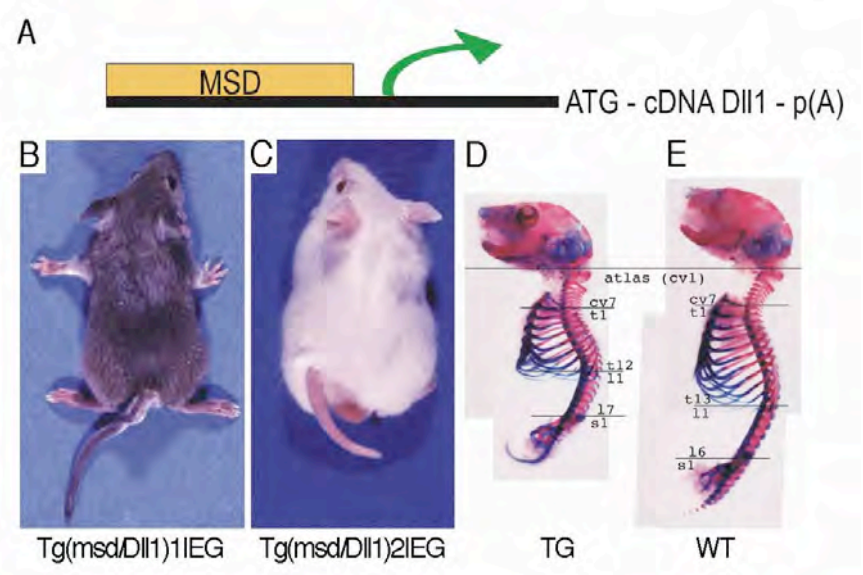

Figure 6: *Mouse line Tg(msd/Dll1)Ieg exhibits a transgenic external and internal phenotype. (A) Scheme of the Tg(msd/Dll1)Ieg transgene vector. (B-C) External phenotype of transgenic mice of (B) line Tg(msd/Dll1)1Ieg and (C) line Tg(msd/Dll1)2Ieg. Both lines show kinked tails and reduced axial length. Skeletal preparations of (D) transgenic and (E) wild-type mice stained with alizarin red and alcian blue. Although the body length is reduced in transgenic mice compared to wild-type littermates, the number of vertebral elements from the first cervical to the first sacral vertebral element remains unchanged.*

number of vertebrae					vertebral fusions					reduced vertebrae				
cv	th	lu	sa	ta	cv	th	lu	sa	ta	cv	th	lu	sa	ta
7	13	6	4	>25	-	-	5-6	-	-	-	-	-	-	2-4
7	**12**	**7**	4	>25	-	-	3-6	4+	+1-4	-	11-12	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	2-3
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-
7	**12**	**7**	4	>25	-	-	-	-	-	-	-	-	-	-
7	13	6	4	>25	-	13+	+1; 5-6	-	-	-	-	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-
7	**12**	**7**	**5**	>25	-	-	1-2; 4-5	-	-	-	11-12	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-
7	**14**	**5**	4	>25	-	-	-	-	-	-	-	-	-	-
7	13	6	4	>25	-	-	3-4	-	-	-	-	-	-	2-4
7	**12**	**7**	4	>25	-	-	4-7	-	-	-	10-12	-	-	-
7	13	6	4	>25	-	-	2-4	-	-	-	-	-	-	-
7	13	6	4	>25	-	-	-	4+	+1	-	-	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	11-14	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-
7	**12**	**7**	4	>25		12+	+1	4+	+1	-	-	-	-	-
7	13	6	4	>25	-	-	-	-	-	-	-	-	-	-

Table 4: *X-ray analyses of the axial skeleton of 18 adult mice without an external phenotype from Tg(msd/Dll1)leg lines. Bold values differ from the wild-type phenotype presented in the bottom row. Numbers with a '+' indicate fusions of vertebral bodies to the next or previous vertebra. "Reduced vertebra" refers to a shortened R/C length of vertebrae. cv, cervical; lu, lumbar; ta, tail; th, thoracic; sa, sacral.*

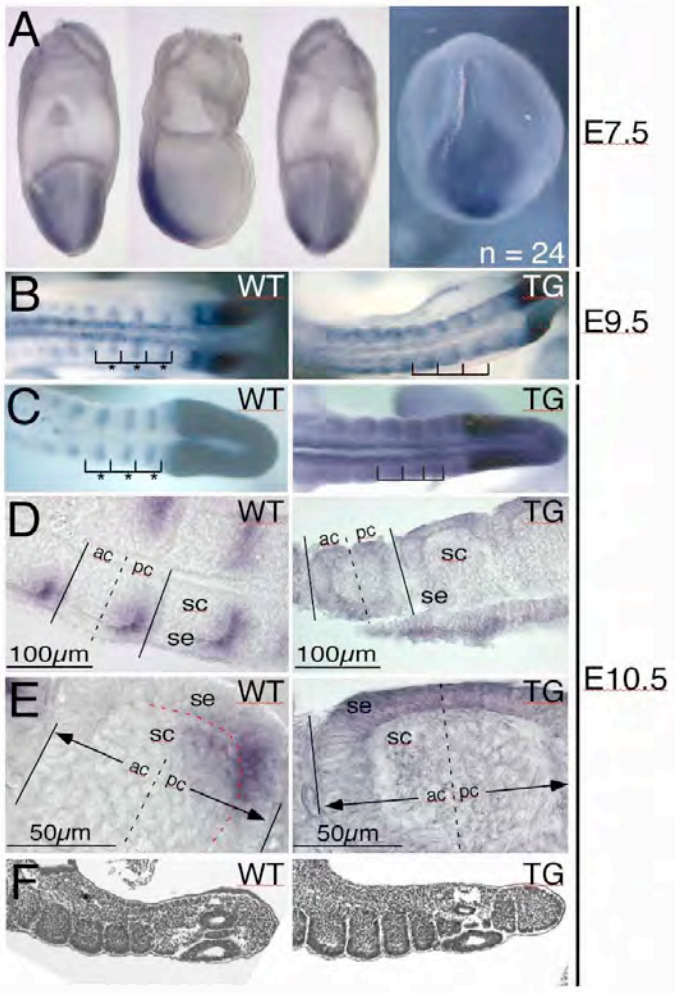

Figure 7: *Dll1 whole-mount RNA in situ hybridisations and somite formation. (A) Dll1 whole mount in situ hybridisations of E7.5 embryos from crosses of transgenic and wild-type mice. 24 embryos were analysed for Dll1 expression patterns. We did not observe differences in Dll1 expression patterns in these embryos. From left to right: view from the cephalic side, lateral view with the cephalic region to the left and the primitive streak side to the right, view from the side of the primitive streak, view onto the node with the primitive streak on top of the node. Each picture was taken from a different embryo. Ectopic Dll1 expression is detected at (B) E9.5 and (C) E10.5 throughout somites in transgenic embryos. Wild-type expression is restricted to caudal somite halves (asterisks). (D, E) Cryo-sections of in situ hybridised E10.5 wild-type embryos reveal expression of Dll1 in the caudal, inner epithelium. A weak staining may be present in cells of the caudal somitocoele. The red broken line in the left panel of (E) indicates the boundary between the somitic epithelium and the inner somitocoele. Transgenic embryos express Dll1 in epithelial cells without caudal restriction. Again, weaker staining is detected in the somitocoele. (F) H/E stained sections of E10.5 embryos reveal normal epithelial somites in wild-type and transgenic embryos. ac, anterior compartment of somite; pc, posterior compartment of somite; sc, somitocoele; se, somitic epithelium; TG, transgenic; WT, wild-type.*

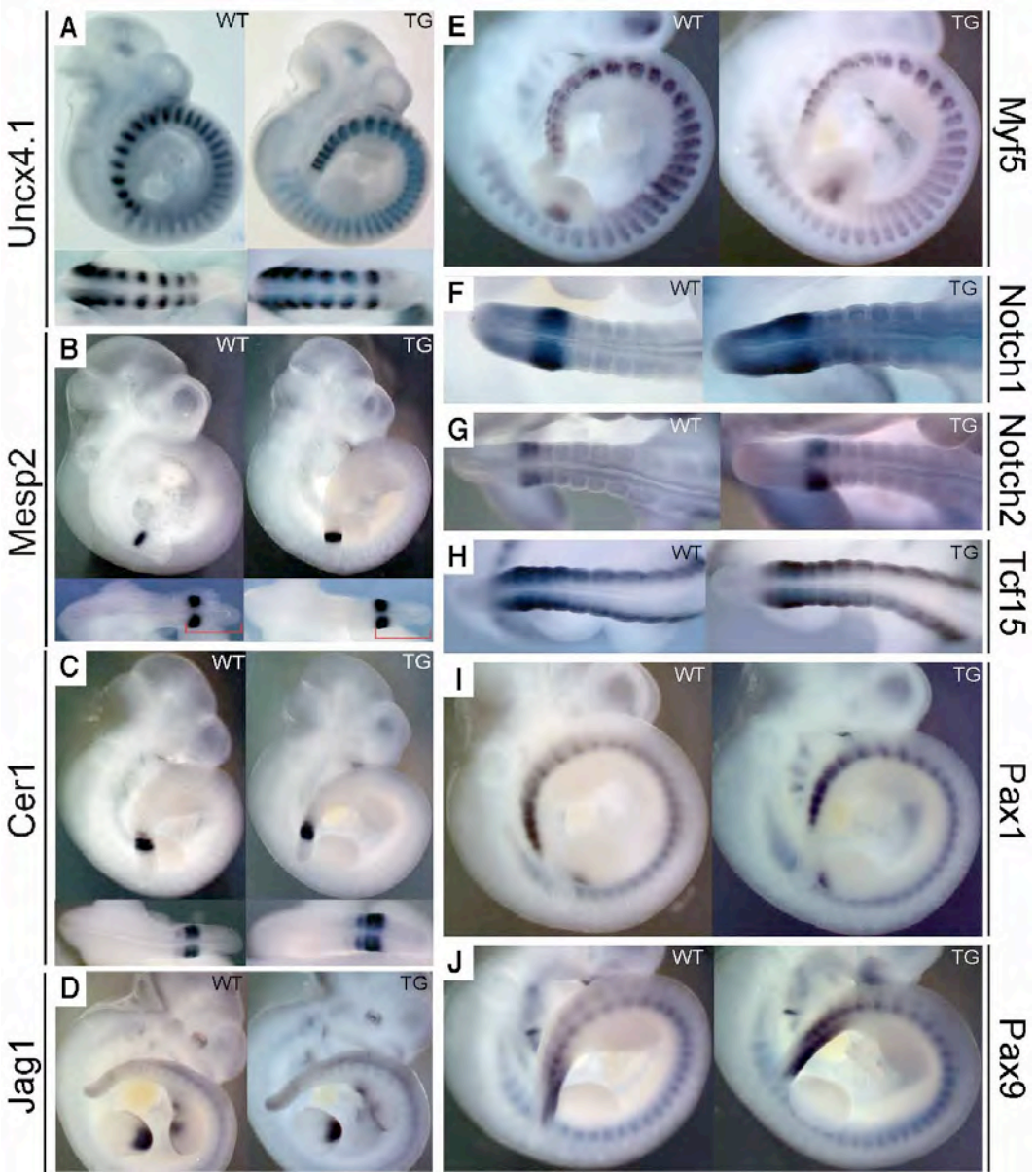

Figure 8: *Expression of Dll1 target and cranial and caudal somite marker genes at E10.5 in Tg(msd/Dll1)Ieg transgenic and wild-type embryos. Top panels in A to C and panels D, E, I, and J show right lateral views of in situ hybridised whole embryos. Lower panels in A to C and panels F, G, and H show dorsal views of the posterior tail regions. The red brackets in the lower panels of (B) indicate the region of the unsegmented, presomitic mesoderm. The gene transcripts to which the in situ probes were specific are indicated next to each panel. We did not detected reproducible changes in gene expression patterns between wild-type and transgenic embryos for the indicated genes at E10.5. TG, transgenic: WT, wild-type.*

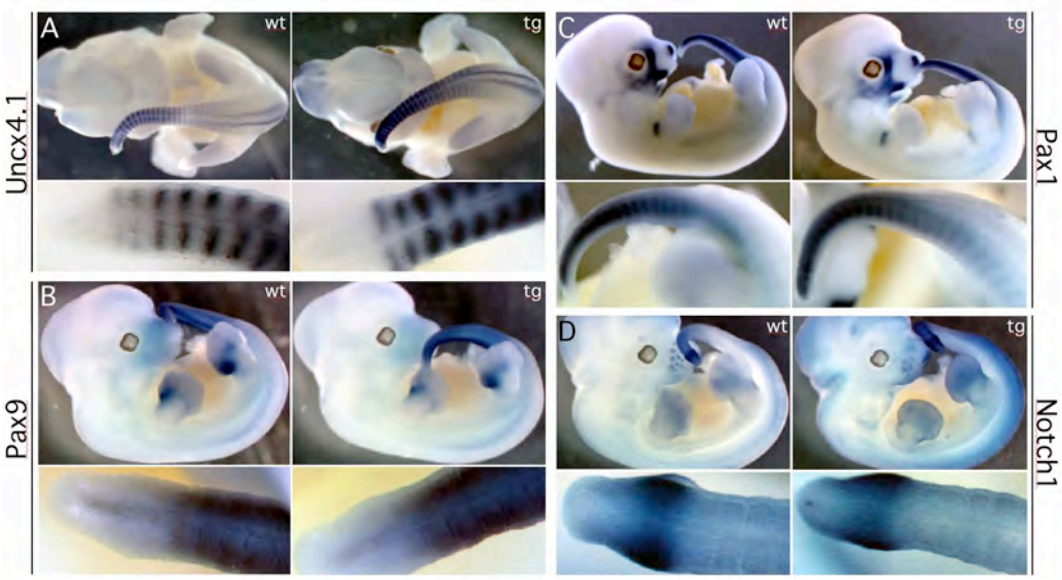

Figure 9: *Whole mount in situ hybridisations of E12.5 wild-type (wt) and transgenic (tg) mouse embryos. In each panel a representative wt embryo is shown on the left and a transgenic embryo is shown on the right. The upper photograph in each panel shows a whole embryo either in a ventral (A) or lateral view (B, C, and D). The photographs below show details from the pre-somitic mesoderm and several pairs of somites; posterior is to the left in the lower photographs. Hybridisation with a probe for Uncx4.1 (A), Pax9 (B), Pax1 (C), and Notch1 mRNA (D).*

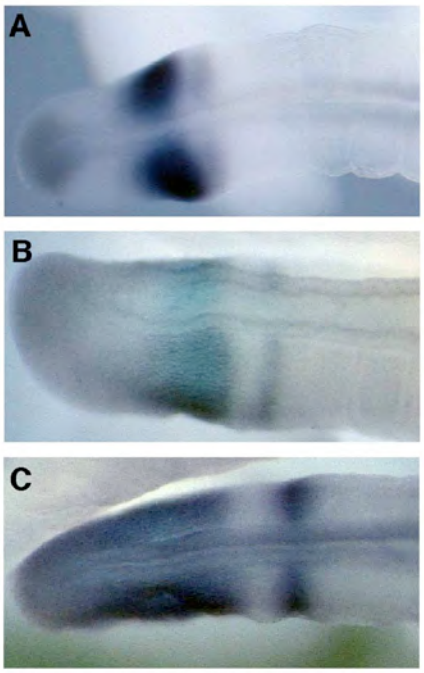

Figure 10: *Three phases of cyclic Lfng expression in the paraxial mesoderm of Tg(msd/Dll1)Ieg embryos at E10.5. (A) After somite formation a weak expression domain is initiated in the tail bud and the recently formed somite, strong expression is detected in the rostral PSM. (B) The strong expression domain is expanded in the PSM and (C) Lfng is expressed in the whole PSM, a weaker domain marks the line of the next somite formation.*

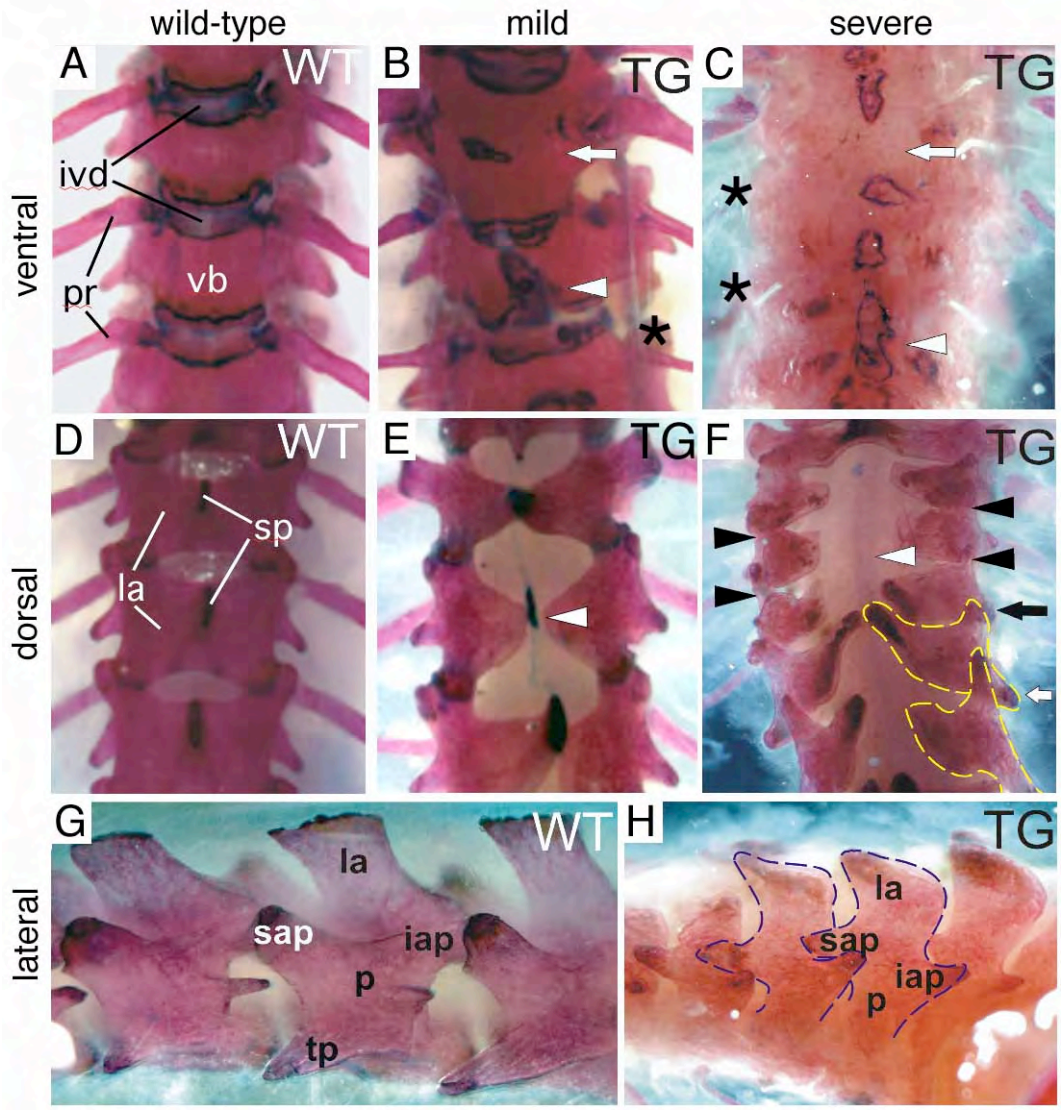

Figure 11: *Alizarin red and alcian blue stained adult skeletons. Alizarin red and alcian blue stained (A-F) thoracic and (G, H) lumbar vertebrae. (B, C) Split vertebrae (arrowhead), fused vertebrae (arrow) and reduced costal heads of ribs (asterisks) compared to (A) wild-type animals are found. (D-F) Dorsal examinations exhibit reduced spinous processes, open neural arches (white arrowheads) and reduced (black arrowheads) or malformed (black and white arrows) intervertebral joints. (G, H) Reduced intervertebral joints occur in (H) transgenic mice compared to (G) wild-types but no fused adjacent neural arches (dashed lines) are observed. iap, inferior articular process; ivd, inter-vertebral disc; la, lamina; p, pedicle; pr, proximal rib; sp, spinous process; sap, superior articular process; TG, transgenic; tp, transverse process; vb, vertebral body; WT, wild-type.*

Ectopic Dll1 expression throughout somite epithelia

Dll1 expression in wild-type and transgenic mouse embryos was examined from E7.5 to E10.5 by whole-mount RNA in situ hybridisation. At E7.5 no differences between wild-type and transgenic embryos were observed (Figure 7A). Ectopic *Dll1* expression in rostral halves of recently formed somites was first detected at E8.0 in transgenic embryos when somitogenesis is initiated and has lasted at least until E10.5 (Figure 7B to E). Variations in the expression level of the transgene were observed but did neither correlate with one of the transgenic lines nor with a specific stage of development. Embryos from whole-mount in situ hybridisations were used for histological sections to determine *Dll1* expressing cells. In wild-type embryos at E10.5, sections revealed significant staining in cells of the somitic epithelium of caudal somite halves (Figure 7D, E). A weak staining was detected in the somitocoele adjacent to the caudal epithelium. In transgenic embryos, *Dll1* expression was also detected in epithelial cells but without restriction to caudal somite compartments (Figure 7D, E). Similar to wild-type embryos a weak staining was detected in the somitocoele.

Epithelial somites appear normal in Dll1 over-expressing embryos

Histological sections of paraffin embedded embryos at E10.5 revealed no differences between somites of wild-type and transgenic embryos (Figure 7F). Nascent somites developed a normal epithelial layer surrounding the somitocoele. The size of somites was identical in five age-matched pairs of transgenic and wild-type embryos between E9.5 and E10.5 in raster electron microscopic images (data not shown). Thus, the over-expression of *Dll1* in the paraxial mesoderm did not affect the early generation of somites with regards to epithelialisation, size and histological appearance at least until E10.5.

We analysed the expression of various rostral and caudal somite marker genes and Notch signalling targets by whole-mount in situ hybridisation of wild-type and transgenic embryos. From E9.5 to E10.5 we found no differences in the expression levels of *Dll3*, *Jag1*, *Notch1*, *Notch2*, *Lfng*, *Uncx4.1*, *Hes5*, *Mesp1*, *Mesp2*, *Paraxis* (*Tcf15*), *Pax1*, *Pax9*, *Myf5*, *Epha4*, *Myog* and *Cer1* (Figure 8A to J, and data not shown) (Barrantes et al., 1999; Biben et al., 1998; Leitges et al., 2000; Mansouri et al., 2000; Saga et al., 1997; Takahashi et al., 2003). The R/C marker and Notch pathway genes *Notch1*, *Notch2*, *Mesp1*, *Mesp2*, *Myf5*,

Uncx4.1, *Pax1*, and *Pax9* were analysed until E12.5 and did not reveal differential gene expression between transgenic and wild-type embryos (Figure 9). Many of these genes also have dynamic patterns of expression in the PSM. In accordance with the normal and regular epithelial somites observed, we did not find evidence for a change in phased gene expression in the PSM of transgenic embryos in comparison to their wild-type littermates. In particular, the cyclic expression of *Lfng* in the PSM (Aulehla and Johnson, 1999; Forsberg et al., 1998) was not affected in Tg(msd/Dll1)Ieg embryos. Distinct phases of Lfng expression were evident in transgenic embryos at E10.5 (Figure 10). Homozygous Tg(msd/Dll1)Ieg mice were generated. Dysmorphologies of the axial skeleton of these mice were not different from the dysmorphologies that were identified in heterozygous transgenic mice. We did, therefore, not undertake a gene expression analysis of homozygous transgenic embryos. We cannot exclude that even higher doses of ectopic *Dll1* expression in the PSM may lead to a different phenotype with regards to cycling gene expression and the generation of R/C polarized epithelial somites.

Dll1 over-expression affects intervertebral articulations and vertebrae morphology

Since the contribution of distinct somitic regions to the elements of the axial skeleton has been rather well described (Aoyama and Asamoto, 2000; Christ et al., 2000; Dubrulle and Pourquie, 2004; Huang et al., 1994), we examined vertebrae morphology with the aim to identify those sclerotome and arthrotome derived cell types that contribute to malformed skeletal elements in *Dll1* transgenic mice. In alizarin red and alcian blue stained skeletons of transgenic mice we observed that structures derived from the somitocoele were frequently missing (Figure 11). In particular, costal heads of ribs were absent or strongly reduced in their thickness (Figure 11B, C; asterisks) and IVDs were often missing resulting in the fusion of vertebral bodies (Figure 11B, C; arrows). The articular processes of the neural arches were either missing in transgenic mice with strong phenotype (Figure 11F, black arrowheads) or reduced and malformed (Figure 11F; black and white arrows). Despite the dysmorphologies of articular processes we did not observe fusions between adjacent neural arches even in transgenic animals with severe phenotype (Figure 11F, H).

Additional dysmorphologies in the axial skeleton of transgenic mice included split vertebral bodies with ectopic pseudo-growth plates (Figure 11B, C; arrowheads) and open neural arches with missing spinous processes (Figure 11E, F; white arrowheads). Pedicles and laminae of neural arches were always present and did not fuse between adjacent vertebrae (Figure 11H).

Developmental progression of axial dysmorphologies

To further characterise the loss of intervertebral joints and midline clefts of the axial skeleton, the developmental progression of this transgenic phenotype was monitored from E12.5 to the newborn stage using alcian blue and alizarin red stainings (Figure 12). Pairwise chondrocyte condensations of the presumptive axial skeleton adjacent to the notochord appeared normal in transgenic embryos at E12.5 compared to wild-type littermates (Figure 12A, B). In wild-type embryos at E13.5 the pairs of cell condensations have come into contact at the axial midline. Here they now form single, rather cylindrical cell condensations that periodically encircle the notochord (Figure 12C). In transgenic embryos at E13.5 pairs of cartilage condensations were not yet merged at the axial midline and regions between successive presumptive vertebrae stained with alcian blue (arrowhead in Figure 12D). In wild-type embryos at E14.5 morphogenesis had proceeded and a regular pattern of strongly alcian blue stained presumptive vertebral bodies and weaker stained presumptive IVDs was evident (Figure 12E). Contrary, in transgenic embryos of the same age, the developing vertebral column stained uniformly with alcian blue (Figure 12F). The extrusion of notochordal cells in transgenic embryos was incomplete such that the rod of axial mesoderm in the regions of vertebral bodies was thicker than in wild-type embryos (Figure 12E, F). In vertebral bodies of newborn wild-type mice, alizarin red staining showed a single, central core of mineralised extracellular matrix (Figure 12G). Mineralisation had progressed also in the early neural arches and morphogenesis had resulted in the formation of superior and inferior articular processes forming the intervertebral joints. In contrast, transgenic newborn mice showed two mineralised cores lateral to the axial midline in each vertebral body (Figure 12H). Intervertebral discs were missing and adjacent vertebral bodies fused. Superior and inferior articular processes were missing in the newborn transgenic mice with severe phenotype (Figure 12H). Thus, the loss of IVDs and

the failure of sclerotome cells to merge at the axial midline was traced back as early as E13.5 in transgenic embryos.

In addition to altered cell differentiation of presumptive IVD cells in transgenic mice, we considered the possibility that the loss of IVD cells might result either from apoptosis of presumptive IVD cells or, alternatively, from over-proliferation of cells of the prospective vertebrae displacing future IVD cells. To analyze these alternatives, we performed Bromodeoxyuridine (BrdU) and TUNEL assays (BrdU: E12.5 (n=4), E13.5 (n=4); TUNEL: E12.5 (n=8), E13.5 (n=3), E14.5 (n=6), E15.5 (n=4) and E17.5 (n=3)). No significant differences between wild-type and transgenic animals were observed in BrdU (Figure 13) and in TUNEL assays (Figure 14) at these developmental stages.

Dll1 over-expression affects chondrocyte hypertrophy and endochondral bone formation in vertebrae

We performed Safranin O stainings on histological sections of paraffin embedded embryos to investigate cartilage development in prospective vertebral bodies (Figure 15). These stainings revealed fewer hypertrophic chondrocytes in vertebral bodies of transgenic rather than of wild-type embryos (E16.5) (Figure 15A, B; arrows). Ossification had progressed normally at E17.5 in wild-type animals but was delayed in transgenic embryos (Figure 15C, D; arrows) and hypertrophic chondrocytes were dorsally displaced (Figure 15D; arrow). RNA in situ hybridisations to detect *Col10a1* gene expression, which is a marker for cells undergoing endochondral ossification (Linsenmayer et al., 1991; Vortkamp et al., 1996), also revealed fewer hypertrophic cells (data not shown).

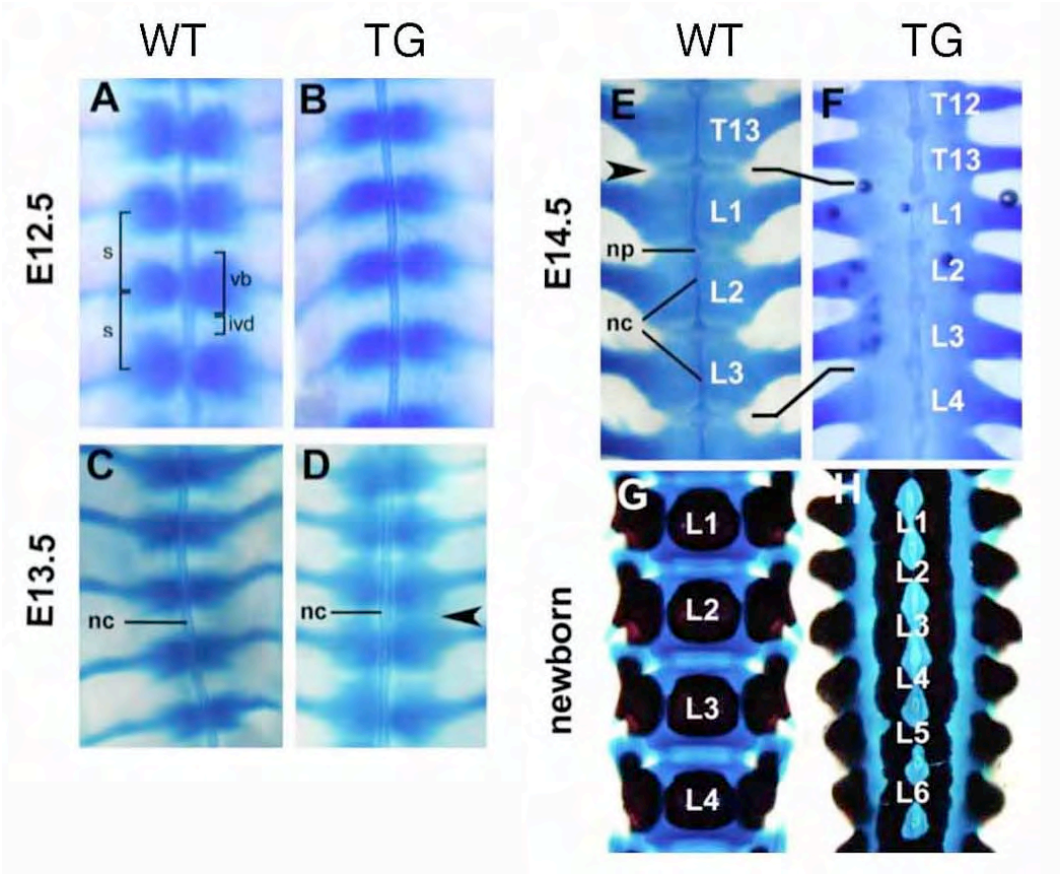

Figure 12: *Developmental progression of the transgenic phenotype in the vertebral column. (A-H) Alizarin red and alcian blue stained wild-type and transgenic lumbar vertebrae, ventral view. (A, B) E12.5 reveals no differences between wild-type and transgenic embryos. (D) At E13.5 presumptive IVDs stain with alcian blue in transgenic embryos (arrowhead) but not in (C) wild-types. At E14.5 (E) vertebrae of wild-type embryos are separated by weaker stained IVDs (arrowhead). The notochord is extruded to the nucleus pulposus. (F) Transgenic vertebrae are shortened, stain uniformly with alcian blue, IVDs are missing and the notochord remains as a rod like structure. Newborn transgenic mice (H) exhibit two lateral centres of ossification compared to one centre in wild-type mice (G). ivd, intervertebral disc; L, lumbar vertebra; nc, notochord; np, nucleus pulposus; s, somite; T, thoracic vertebra; TG, transgenic mouse; vb, vertebral body; WT, wild-type.*

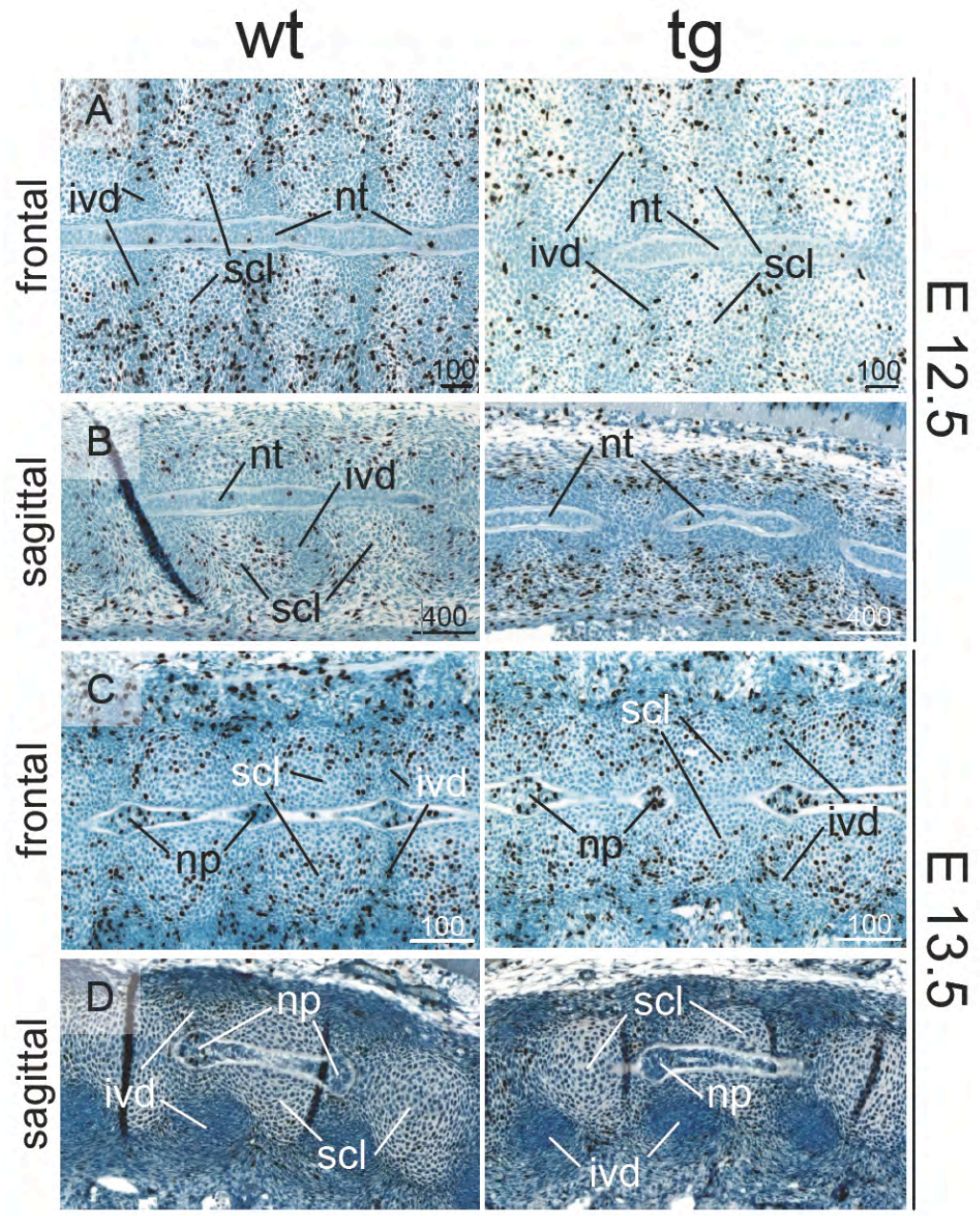

Figure 13: *BrdU assay in frontal (A and C) and mid-sagittal sections (B and D) of E12.5 and E13.5 wild-type (wt, left) and transgenic (tg, right) embryos. (A) shows frontal sections at the level of the notochord (nt) through a wt (left) and transgenic (right) embryo at E12.5. Presumptive regions of intervertebral discs (ivd) and the region of the sclerotome (scl) can be histologically distinguished. There is no difference in the number of BrdU labelled cells if regions of ivd and scl are compared. The sectioned transgenic embryo (right) shown in panel (B) has a severe phenotype: There is no clear metameric sequence of ivd and scl regions. At E13.5 (C and D) the formation of the nucleus pulposus (np) initiates. Also at this stage there is no change in the number of BrdU labelled cells if scl and ivd are compared in wt and tg embryos. Numbers above scale bars indicate the size in µm.*

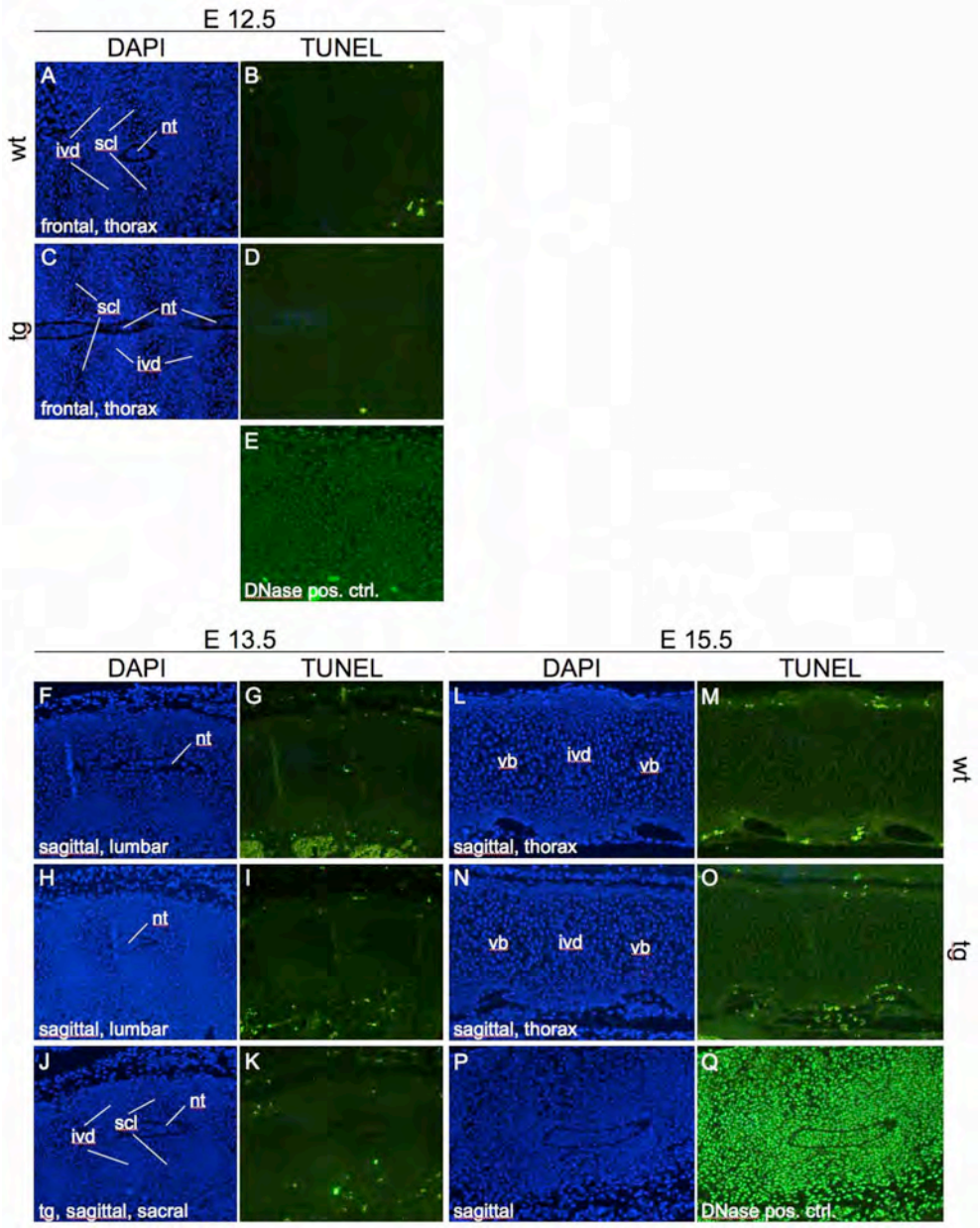

Figure 14: *TUNEL assay and DAPI stainings in sections of E12.5 (A - E), E13.5 (F - K), and E15.5 (L - Q) wild-type (wt) and transgenic (tg) embryos. Panels (A - D) show frontal sections at the level of the notochord in the thoracic region. Notochord (nt), sclerotome (scl), and presumptive intervertebral regions (ivd) are histologically recognizable in DAPI stainings (left panels). Based on TUNEL assays (right panels, B and D) no indication of increased DNA fragmentation was evident in any particular region of the section. Panel (E) shows a positive control that was DNase treated. Sagittal sections through the lumbar (F - I) and sacral (J, K) regions did not reveal increased fluorescence in particular regions in the TUNEL assay in wt (F and G) and transgenic (H - K) embryos at E13.5. Panels (L - O) show sagittal sections through the thoracic regions of a wt (L, M) and a transgenic (N, O) E15.5 embryo. The presumptive vertebral bodies and a future ivd are discernible in the*

DAPI stainings. No increased fluorescence was detected in these regions in the TUNEL assay. Panel (Q) shows a DNase treated positive control for the TUNEL assay and panel (P) is a DAPI staining of the same section.

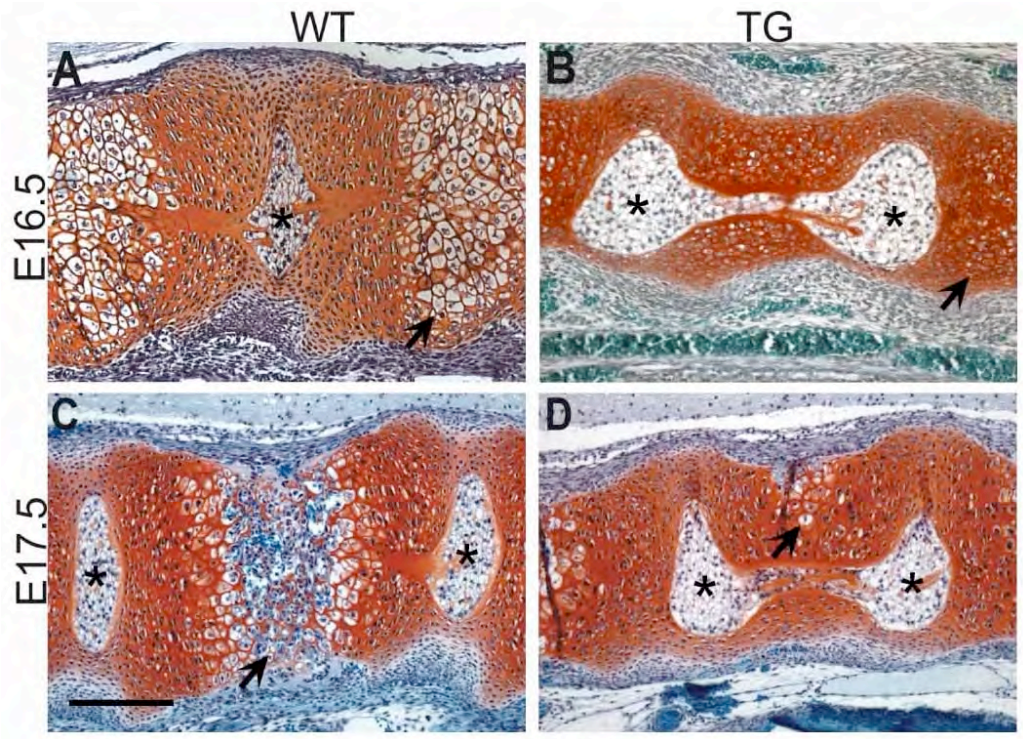

Figure 15: *Safranin O stainings for cartilage of mid-sagittal sections through the presumptive vertebrae of transgenic and wild-type embryos. (A) In wild-type embryos at E16.5 hypertrophic chondrocytes are observed in the centre of the vertebrae (arrow). (B) The number of chondrocytes undergoing hypertrophic differentiation is reduced in transgenic embryos (arrow). At E17.5 (C) chondrocytes of wild-type embryos lose the cartilage matrix and vascularisation and invasion of osteocytes starts (arrow). The notochord is extruded from the vertebrae. (D) In transgenic vertebrae of the same age fewer cells are hypertrophic and are dorsally displaced (arrow). Ossification is delayed and notochord extrusion is incomplete. Scale bar: 200 µm. Asterisks: nucleus pulposus*

Discussion

Mouse *Dll1* expression in the PSM and caudal somite halves, is necessary for the formation of epithelial somites and maintenance of caudal somite identity (Bettenhausen et al., 1995; Hrabe de Angelis et al., 1997). Two transgenic mouse lines (Tg(msd/Dll1)Ieg) over-expressing *Dll1* in the paraxial mesoderm including rostral somite compartments under the control of the msd *cis*-regulatory element were generated (Beckers et al., 1999; Cordes et al., 2004). We investigated the role of restricted, compartmentalised *Dll1* expression for the maintenance of R/C somite identity and the morphogenesis of the axial skeleton. Tg(msd/Dll1)Ieg animals were viable and fertile. They developed normal epithelial somites and gene expression analysis on R/C marker genes revealed, that the uniform over-expression of *Dll1* throughout the somites was not sufficient to confer caudal compartment identity to rostral somite halves. Nevertheless transgenic animals had at least three distinct phenotypic alterations of the vertebral column: (i) arthrotome related dysmorphologies, (ii) midline defects and (iii) a failure in chondrocyte maturation. In the following, the potential role of *Dll1* over-expression for these phenotypic traits and the requirement of caudally restricted *Dll1* expression for intervertebral joint formation are discussed.

Somite formation and rostro-caudal polarity are not affected by Dll1 over-expression

Dll1 deficient embryos (Dll1$^{tm1Gos/tm1Gos}$) showed severe patterning defects in the paraxial mesoderm and died before E12.5 (Hrabe de Angelis et al., 1997). In these mutants, the R/C pattern of somites was not established and maintained as in wild-type embryos and nascent somites did not epithelialise normally. The requirement of *Dll1* for the formation of the axial skeleton was also studied in a mouse model expressing a truncated and dominant-negative version of *Dll1* (Dll1^{dn}) under the control of the msd cis-regulatory element (Tg(msd/Dll1^{dn})) (Cordes et al., 2004). In the axial skeleton such transgenic mice exhibited fusions of laminae of neural arches, reduction or loss of pedicles, split vertebral bodies as well as rostral homeotic transformations at the cervical-thoracic transition. Gene expression analyses revealed a reduction of *Uncx4.1* expression in caudal compartments and an expansion of the expression domain of the rostral somite marker gene *Tbx18*. These data suggested a partial loss of caudal somite

compartment characteristics in Tg(msd/Dll1^{dn}) animals. Despite the fact that Tg(msd/Dll1)Ieg animals exhibited dysmorphologies of the axial skeleton, *Dll1* transgenic embryos did not display changes in R/C marker gene expression.

The midline defects in the axial skeleton, the vertebral fusions and dysmorphologies of ribs observed in Tg(msd/Dll1)Ieg animals were reminiscent of somitogenesis related phenotypes observed in other mutant mice. All of these mutant animals are, however, characterised by alterations of R/C marker gene expression. For example, the bilateral centres of ossification in the vertebral bodies of *Paraxis* (Blanar et al., 1995; Burgess et al., 1995; Burgess et al., 1996; Sosic et al., 1997), *Dll3* (Dunwoodie et al., 2002; Kusumi et al., 1998; Shinkai et al., 2004) and *Psen1* (Saga et al., 1997; Shen et al., 1997; Takahashi et al., 2000; Wong et al., 1997) deficient mice as well as missing spinous processes in *Uncx4.1* (Leitges et al., 2000; Mansouri et al., 2000) and *Dll3* deficient skeletons resemble the midline defects found in the *Dll1* over-expressing mice. Proximal rib malformations and/or rib fusions occur consistently in *Paraxis*, *Psen1*, *Dll3* and *Uncx4.1* as well as in the *Dll1* over-expressing transgenic mice. Fusions of vertebral bodies or precursors along the R/C axis and the loss of IVDs as described for Tg(msd/Dll1)Ieg animals were also found in *Paraxis*, *Psen1* and occasionally in *Uncx4.1* deficient mice. Our analysis of the Tg(msd/Dll1)Ieg mice clearly shows that these phenotypes can occur in mice with apparently normal R/C polarity of somites. The fact that rostral and caudal sclerotome derived structures were not missing in the Tg(msd/Dll1)Ieg mice additionally supports the hypothesis that R/C somite polarity is not affected and suggests an alternative origin of the axial dysmorphologies. A potential reason for the finding that transgenic Dll1 over-expression does not affect the polarity of somites maybe that the endogenous expression of *Dll3* inhibts the ectopic activation of Notch signalling in anterior somite compartments (Ladi et al., 2005).

Dll1 over-expression might affect arthrotome cell differentiation

In addition to midline defects, we observe the loss or reduction of IVDs, articular (zygapophyseal) joints and proximal ribs in the axial skeletons of Tg(msd/Dll1)Ieg animals. These phenotypic characteristics are present despite normal epithelialisation and R/C polarity in transgenic mice. One possible explanation for the transgenic phenotype affecting the intervertebral joints may be that the loss of restricted *Dll1* expression results in a late defect in re-segmentation of

sclerotomal compartments. Alternatively, we noted that all the vertebral structures (IVDs, articular joints, and proximal ribs) that are affected in *Dll1* over-expressing mice are located in a central position of the vertebral motion segment. Injection of single somite cells with fluorescent dye (Wong et al., 1993) and homotopical grafting experiments of quail and chicken somitocoele cells (Huang et al., 1996; Huang et al., 1994) had previously suggested that somitocoele cells form a distinct somitic compartment, the arthrotome. During later development these cells are located at a central position of the vertebral motion segment forming vertebral joints, IVDs and the proximal ribs (Christ et al., 2004). Accordingly, the microsurgical removal of somitocoele cells from chick epithelial somites and preventing the epithelial cells from contributing to the somitocoele cell population, resulted in the loss of IVDs, fusion of vertebral bodies and the absence of intervertebral joints (Mittapalli et al., 2005). These analyses on the immediate fate of somitocoele cells have consistently been performed in the avian system (Brand-Saberi et al., 1996; Huang et al., 1997; Huang et al., 1994; Wong et al., 1993). A related study in mice showed *Pax1* expression in somitocoele cells and the ventromedial sclerotome. Mice deficient for the *Pax1* gene lack derivatives of the ventromedial sclerotome or have reduced IVDs, proximal ribs and articular processes (Wallin et al., 1994). Considering the experimental evidence on the fate of somitocoele cells in the avian embryo and of *Pax1* expressing cells in mice, we propose the hypothesis that the observed reduction or loss of central structures of the vertebral motion segment may be due to a failure in the development or specification of the arthrotome in Tg(msd/Dll1)leg transgenic embryos. However, we did not observe any changes in the expression of *Pax1* in transgenic embryos until E12.5. This observation together with the normal histological appearance of somites suggests that the *Dll1* over-expression throughout somites and the PSM does not affect the initial formation of somitocoele cells. Instead we consider the possibility that the over-expression of *Dll1* in epithelia of somites might affect the contribution of epithelial cells to the somitocoele (Mittapalli et al., 2005; Wong et al., 1993).

Dll1 over-expression negatively regulates chondrocyte differentiation

Tg(msd/Dll1)leg animals exhibited a delay in chondrocyte hypertrophy and endochondral bone formation in presumptive vertebral bodies. Mid-sagittal as well as lateral-sagittal sections through the vertebral column of transgenic

embryos at E16.5 and E17.5 displayed less hypertophic chondrocytes that undergo endochondral ossification than in wild-type embryos (Figure 15). Previous lacZ reporter gene analyses in *Dll1*$^{+/tm1Gos}$ animals (Hrabe de Angelis et al., 1997) revealed no *Dll1* expression in presumptive vertebral bodies, IVDs or the notochord (Beckers et al., 1999). We therefore assume that chondrocyte maturation was inhibited in transgenic embryos due to the earlier sclerotomal over-expression of *Dll1*. These observations essentially confirm previous studies that have revealed that Delta/Notch signalling acts as a negative regulator for the transition from pre-hypertrophic to hypertrophic chondrocytes (Crowe et al., 1999; Dong et al., 2006; Watanabe et al., 2003).

Conclusion

In conclusion, the over-expression of the *Dll1* gene in rostral epithelial somites was not sufficient to confer caudal somite identity to rostral compartments in transgenic embryos. It had been suggested previously that the compartmentalised expression of *Dll1* later in epithelial somites might be necessary for the maintenance of segment boundaries. However, it has been unclear so far, through which biological process *Dll1* could function to maintain segment boundaries. Our data from transgenic mice over-expressing *Dll1* suggest that the restricted *Dll1* expression in caudal epithelial somites may be required for the proper development of the arthrotome compartment. The failure of arthrotome development in Tg(msd/Dll1)Ieg mice leads to distinct dysmorphologies of the central region of the vertebral motion segment. The mechanism through which *Dll1* acts to specify cells of the arthrotome still needs to be elucidated.

The work presented in the chapter, 3.3.6, is extracted from our manuscript by Teppner *et al.*, which is currently in press in the journal BMC Developmental Biology.

Perspectives

Two current projects continue previous work on the *Dll1* gene regulation and function. First, we are analysing the endogenous function of the mesodermal (*msd*) regulatory element in vivo, in its endogenous position by targeted mutagenesis (3.3.7). Second, following the identification of novel *Dll1* target genes by expression-profiling and in situ hybridisation (see above, 3.3.5), we are

undertaking the next logical step towards the functional analysis of particularly interesting potential target genes. We have begun to persue this strategy by generating a series of novel mutant alleles of the *Ifitm1* gene (3.3.8).

3.3.7. The in vivo functional requirement of the *msd* cis-regulatory element. Regulatory functions here and there in the genome.

In our previous work we had identified a 1,6 kb mesodermal cis-regulatory element, termed *msd*, located approximately 2 kb up-stream of the *Dll1* coding sequence. This *msd* enhancer was sufficient to direct expression of a *lacZ* reporter gene in the pre-somitic mesoderm and somites from E7 to at least E13.5 of transgenic mice (Beckers et al., 2000). We are now addressing the question whether the *msd* regulatory region also is required for normal expression of the *Dll1* gene. For this, we have generated two alleles by gene targeting in mouse embryonic stem (ES) cells (Figure 16): In the first allele ($Dll1^{tm1leg}$), the msd element was exchanged against a hygromycin resistance cassette. In the second allele ($Dll1^{tm1.1leg}$), *msd* was deleted using *Cre* mediated recombination leaving behind a single *loxP* site in the place of the endogenous *msd* regulatory element. We have generated mice that are homozygous for either the *msd* replacement ($Dll1^{tm1leg}$) or the *msd* deletion allele ($Dll1^{tm1.1leg}$). Both mouse lines are viable and fertile. Offspring from heterozygous crosses are born with genotypes according to Mendelian inheritance, suggesting that the mutant alleles do not affect embryonic lethality.

The msd deletion and replacement alleles have overlapping but distinct phenotypic alterations.

To analyse the consequences of the *msd* deletion and replacement on the morphology of the vertebral column we are analysing alizarin red and alcian blue stained skeletons of newborn wild-type, and heterozygous and homozygous mutant animals. Our preliminary analysis suggests that 4 types of dysmorphologies in the axial skeleton can be distinguished: (i) an ectopic rib attached to the 7^{th} cervical vertebra, potentially representing a posterior homeotic transformation, (ii) split vertebral bodies, (iii) fusions of vertebral bodies, and (iv) fusions of neural arches. Whereas the homeotic transformation (Figure 17) and the split vertebral bodies are present in both *msd* alleles, the fused neural arches and fused vertebral bodies are evident with strongly reduced penetrance in the

msd deletion allele when compared to the *msd* replacement allele. Thus, we are currently pursuing the hypothesis that the phenotypic alterations evident in both alleles may result from genuine *msd* functions. On the other hand the additional *msd* replacement phenotypic alterations may be due to the presence of the hygromycin resistance gene and the promoter of the selection cassette in the vicinity of the *Dll1* gene. As one possibility, these ecotpic sequences may affect the regulation from the endogenous *Dll1* promoter.

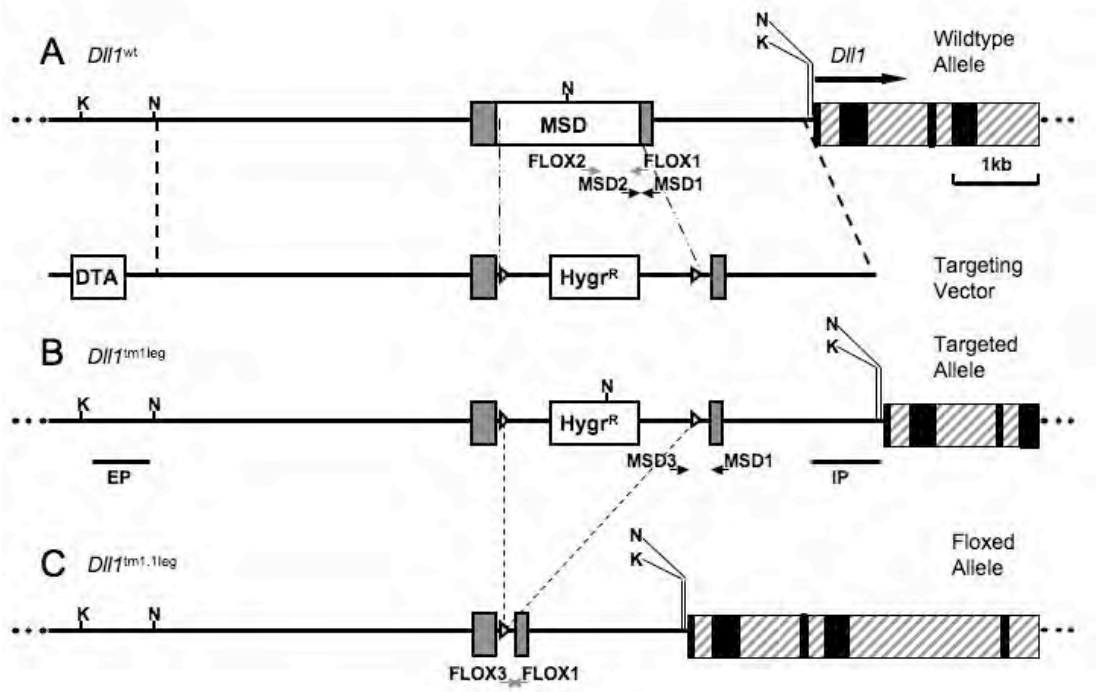

Figure 16: *Replacement and deletion of the msd regulatory region.* ***(A)*** *shows the Dll1 wild-type allele and part of its up-stream region. Below, the targeting vector for the replacement of the msd regulatory region against the hygromycin resistance cassette is indicated. The large arrow indicates the orientation of Dll1 transcription.* ***(B)*** *By homologous recombination in mouse ES cells the* $Dll1^{tm1leg}$ *allele was generated in which the msd region is replaced against the selection marker cassette.* ***(C)*** *Following Cre/loxP mediated recombination the msd region was deleted in the* $Dll1^{tm1.1leg}$ *allele. Black boxes indicate Dll1 exons, grey striped boxes indicate Dll1 introns, grey boxes indicate Dll1 neural enhancers. Small arrows indicate primers used for PCR genotyping (black: msd replacement, grey: msd deletion). K, Kpn I; N, Nco I; loxP sites (grey triangles); thick lines indicate probes internal (IP) and external (EP) with regards to the targeting vector used for Southern blot analysis.*

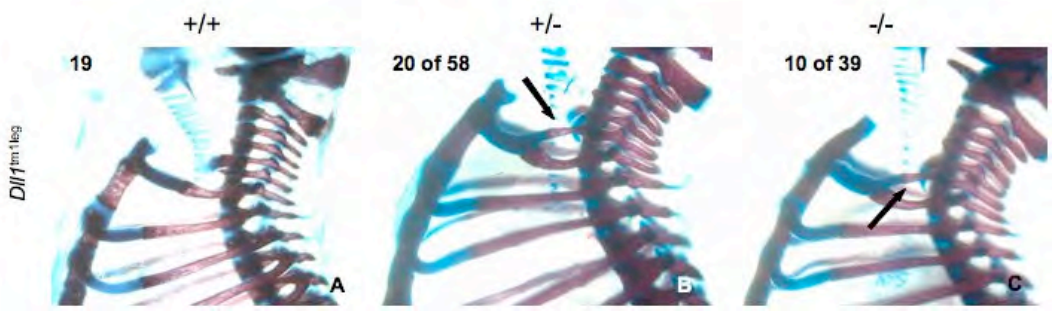

Figure 17: *Alizarin red and alcian blue staining of newborn (A) wild-type, (B) heterozygous and (C) homozygous Dll1^{tm1leg} mice. Both, Dll1^{tm1leg} heterozygous and homozygous, show a posterior homeotic transformation of the 7th cervical vertabra with similar penetrance.*

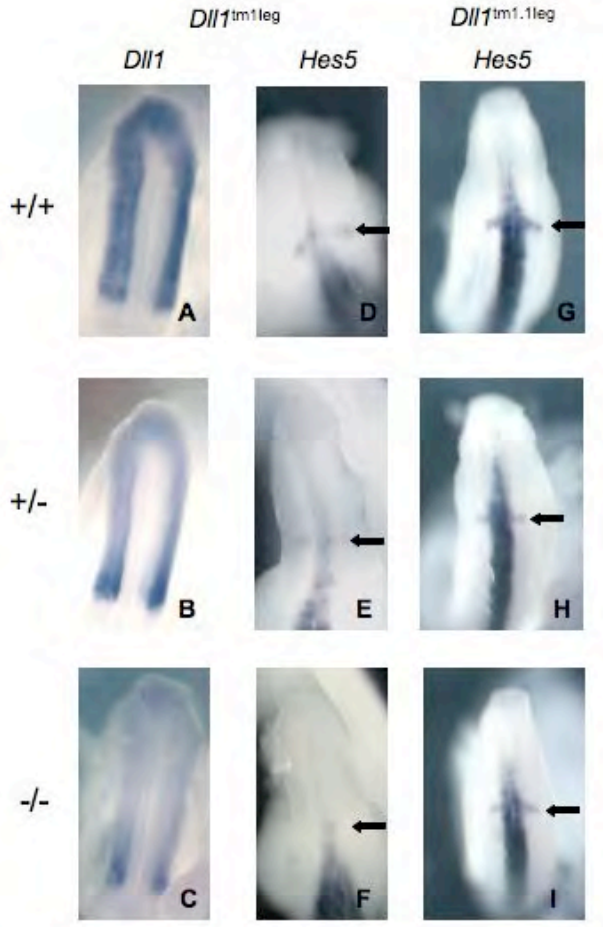

Figure 18: *Dll1* and *Hes5* whole mount in situ hybridisations in wild-type and *msd* mutant embryos (E9,5). Pictures show the pre-somitic mesoderm (psm); arrows point to expression domains of *Hes5* in the psm.

Msd is not necessary for mesodermal Dll1 expression.

We have begun to analyse the expression of the *Dll1* gene in embryos carrying both *msd* alleles. From our preliminary data, it appears that *Dll1* expression in the pre-somitic mesoderm (psm) is reduced in $Dll1^{tm1leg}$ heterozygous and homozygous embryos at E9,5 (Figure 18A to C). Accordingly, we also observe reduced expression of the target gene *Hes5* in the psm of these mutant embryos (Figure 18D to F). In contrast, in $Dll1^{tm1.1leg}$ homozygous mutant embryos of the same developmental stage *Hes5* is apparently expressed as in wild-type embryos (Figure 18G to I). This finding further supports that both *msd* alleles cause different phenotypes also at the molecular level. The preliminary data, in addition, demonstrates that *Dll1* is expressed in the paraxial mesoderm also in the absence of the *msd* regulatory region. Thus, *msd* is sufficient to direct transgenic expression of artificial genes (at least *lacZ*, *Cre*, the *Dll1* and $Dll1^{dn}$ cDNA) into the paraxial mesoderm in a way that is reminiscent of the endogenous *Dll1* expression. Yet, *msd* is not necessary for the endogenous expression of *Dll1* in the paraxial mesoderm. We are currently pursuing a detailed analysis of *Dll1* expression throughout development in both *msd* alleles to dissect the role of *msd* for *Dll1* gene regulation. Subsequently, we will determine the developmental origin of the observed dysmorphologies in the axial skeleton.

3.3.8. Functional studies of novel, putative *Dll1* targets.

We have selected one of the newly identified *Dll1* target genes for gene functional analyses (Machka et al., 2005). In addition to generating a loss-of-function allele of the *Ifitm1* gene by homologous recombination in mouse ES cells, we have also taken the strategy to identify potential hypomorphic alleles using a gene-driven ENU mutagenesis screen. For this, we have sequenced three exons of the *Ifitm1* gene in 20.000 ENU mutagenised genomes. We identified 5 mutations in the coding sequence (Figure 19) and selected one mutation to re-derive life mice from archived sperms. The selected D34V non-conservative exchange of an aspartic acid into the hydrophobic residue valine is located in a conserved CD225 domain (Figure 20). Mice carrying the mutation were born and are currently bred for subsequent phenotyping. Results from

knock-down experiments suggest the requirement of *Ifitm1* for normal somitogenesis (Lickert et al., 2005).

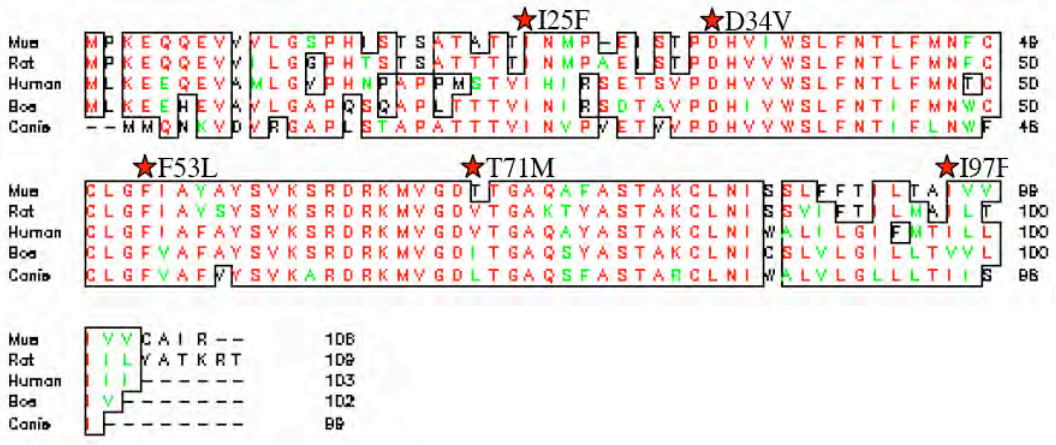

Figure 19: *Amino acid exchanges caused by 5 ENU induced mutations in the Ifitm1 coding region identified by sequencing an archive of approximately 20.000 F_0 founder genomes.*

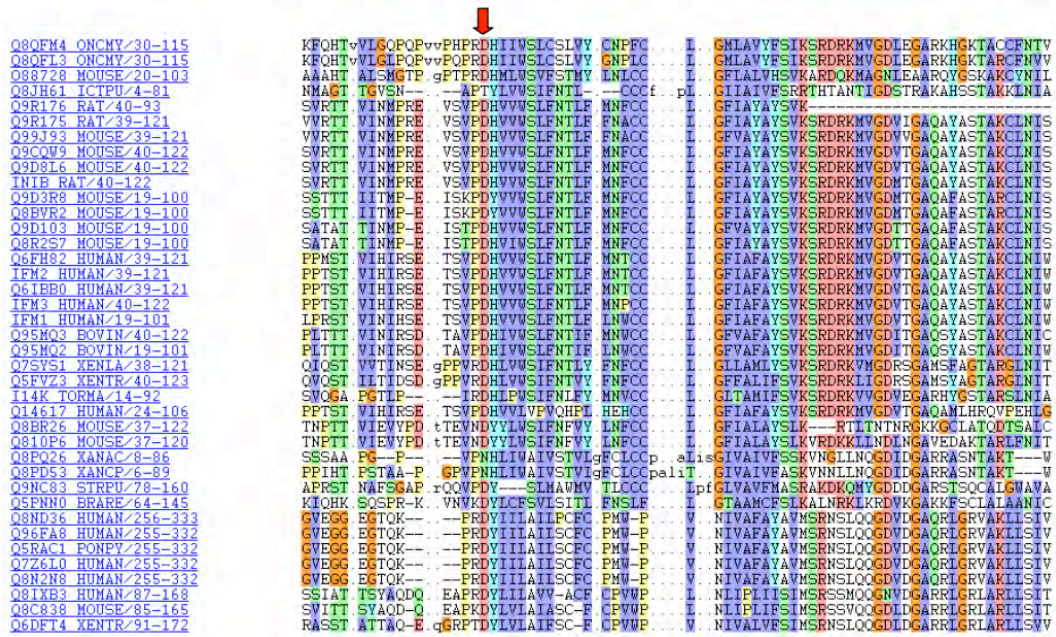

Figure 20: *According to the PFAM database the Ifitm1 protein contains the CD225 domain. The alignment of CD225 domain containing proteins shows that the aspartic acid at position 34 of the Ifitm1 protein (arrow) is highly conserved in these proteins. The mutation D34V changes the acidic residue aspartic acid (D) into the hydrophobic residue valine (V) and might strongly influence protein function.*

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6. SHORT CURRICULUM VITAE

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1987 - 1988	Military services in Hammelburg, Bad Mergentheim, and Koblenz.
1988 - 1993	Studies of Biology at the Ruprecht-Karls University, Heidelberg

Professional education and experience:

1992 - 1993	Diploma thesis at the EMBL, Heidelberg, “Regulation of the human CRP gene in transgenic mice”
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7. LIST OF AUTHOR'S PUBLICATIONS

7.1. Original articles and reviews

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7.2. Book chapters

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8. ATTACHMENT: REPRINTS OF SELECTED PUBLICATIONS

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