Med12 is an essential coordinator of gene regulation during mouse development

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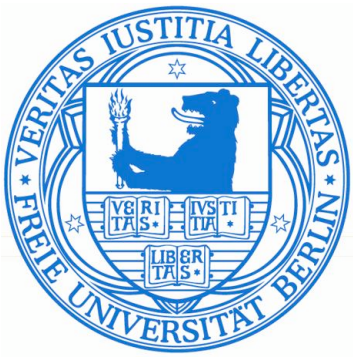

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1 An introduction to eukaryotic transcription and the Mediator

Transcriptional regulation is arguably the most important step controlling the decision of which genes are to be expressed at a given time. (Orphanides and Reinberg, 2002; Panning and Taatjes, 2008). It is now well established that cells have a large plethora of tools with which to control recruitment of RNA polymerases (Pol) and associated general transcription factors (GTFs) to the promoters of genes and regulate transcription during its distinct processes i.e., initiation, elongation and termination (Kornberg, 2007; Levine and Tjian, 2003).

Eukaryotes have three distinct RNA polymerases with different specificities towards the classes of genes that each transcribes. Pol I and Pol III transcribe a limited number of genes encoding ribosomal, transfer and small nuclear RNAs. Protein coding and micro RNA genes are transcribed by Pol II (Sikorski and Buratowski, 2009) and it is the regulation of Pol II activity that is the main topic of this thesis. Each of the polymerases has different associated GTFs. For Pol II these include, transcription factor IIA (TFIIA), TFIIB, TFIID, TFIIE and TFIIH (Figure 1.1). Thus, eukaryotic transcription requires several proteins, associated in a large complex, the composition of which depends on the transcribed gene. This provides the first level of control over transcription available to eukaryotes (Krishnamurthy and Hampsey, 2009).

Second, DNA in eukaryotes is found in a chromatin state, wrapped around nucleosomes. Nucleosomes are composed of histones and can be covalently modified. This influences their position on the DNA and the degree to which chromatin is condensed. In addition, DNA can itself be covalently modified also contributing to modulate chromatin conformation. Ultimately, chromatin structure regulates the binding of proteins that impinge on transcription and the actual process of transcription (Mellor, 2005).

Finally, transcription factors (TFs) are possibly the strongest and most direct influence driving transcription and consequently cell-fate decisions. TFs bind directly to DNA and control transcription by modulating the recruitment of polymerase machinery components and their activity (Kadonaga, 2004). Sequence-specific binding sites for TFs can be found in close-proximity to the transcriptional start site (TSS) or up to hundreds of thousands base-pairs away (Sandelin et al., 2007). Many TFs have a restricted expression pattern that leads to transcription of its target genes in very precisely defined cell-types and/or tissues. The combinatorial mode of action of TFs, whereby a gene can be targeted by several TFs, dramatically increases the complexity available for the fine-tuning and specificity of gene expression (Remenyi et al., 2004).

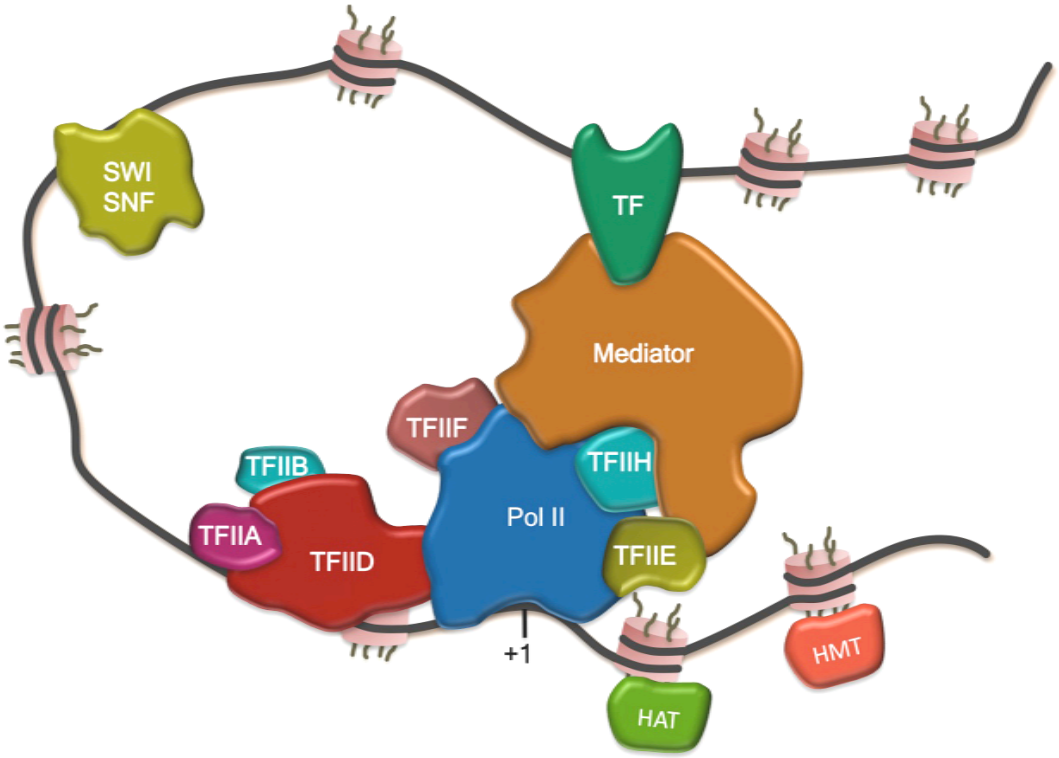

Figure 1.1 Proteins involved in gene transcription

At an active promoter of a protein-coding gene the following protein complexes can be found. RNA polymerase II and associated cofactors are responsible for the actual transcription process and assemble close to the transcription start site (+1), the SWI/SNF complex controls position of nucleosomes (in pink). Proteins with HAT and HMT activities can modify the tails of histones (black lines) and the Mediator is responsible for bridging transcription factors (TF) and the polymerase machinery. Further definition of abbreviation can be found within the text in the current and following sections.

1.1 Coregulators of eukaryotic transcription

Besides TFs, Pol II and the GTFs, other protein complexes are necessary to regulate eukaryotic transcription. Transcriptional coregulators function both as coactivators and as corepressors depending on the positive or negative effect they have on gene transcription. They can act by remodeling chromatin structure and controlling DNA accessibility, or by directly influencing the activities of the transcriptional machinery. Some of these essential proteins are present only in specific cell types and therefore have a direct influence on the gene expression programs of certain tissues (Roeder, 2005).

One of the most prominent co-regulatory complexes is the SWI/SNF (SWItch/Sucrose NonFermentable) complex, which, in an ATP-dependent fashion, is able to remodel and shuffle nucleosomes changing DNA accessibility to TFs and the transcription machinery. During the actual process of transcription, this complex is also required to make the DNA template available to be read by polymerases (Simone, 2006).

Also included in the class of coregulators that impinge on transcription are proteins that modulate the chromatin structure by introduction of covalent modifications on the residues of the histone tails (Taatjes et al., 2004a). These modifications are able to create an “open”, favorable transcriptional environment, or a “closed” status, silencing genes and even whole genomic regions (Bernstein et al., 2007). Among the better-studied modifications is the introduction of positively charged acetyl groups by proteins with histone acetyltransferase (HAT) activity, a positive mark for transcription. Methylation of histone residues by histone methyltransferases (HMTs) also influences transcription but in a more ambivalent fashion according to the residues that are modified (Kouzarides, 2007). Some proteins can “read” the modifications present on the histone residues and then, accordingly, recruit further modifiers of chromatin leading to a fully activated or repressed status of the chromatin. Proteins with the dual activities i.e., reading and modification, can sometimes be found clustered in one complex, as it is found in the Polycomb

complex, first identified in *Drosophila melanogaster* but whose importance extends to mammals where it is a crucial developmental regulator (Simon and Kingston, 2009).

One other class of coregulators contains DNA methyltransferases (DNMTs), which are proteins capable of methylating CpG dinucleotides in the genome. The traditional and arguably most important role of this modification is that of transcriptional repression whereby this modification can help the establishment of a heterochromatin domain with a high degree of DNA compaction leading to the repression of transcription (Law and Jacobsen, 2010).

Coregulators that have a more direct influence on transcription not only act by helping recruitment of the RNA polymerase and/or GTFs to the proximal promoter of genes establishing the pre-initiation complex (PIC), but are also able to activate the transcriptional machinery leading to elongation and productive transcription (Roeder, 2005). TFIID and p300 are among such coregulators (Taatjes et al., 2004a). Similar examples are known, but none other works in such a conserved and universal fashion as the multi-protein Mediator complex which functions as a molecular bridge between transcription factors bound at the promoters of their target genes and the polymerase machinery at the proximal promoter of these targets (Bjorklund and Gustafsson, 2005; Kim and Lis, 2005; Malik and Roeder, 2005). A schematic representation of some of the intervening proteins assembled on the promoter of an active gene can be seen in Figure 1.1.

1.2 The Mediator complex

The Mediator was first discovered in the yeast *Saccharomyces cerevisiae* by the Kornberg and Young laboratories, via biochemical purification of nuclear proteins capable of inducing transactivation and following a genetic screen for suppressors of a Pol II mutation, respectively (Flanagan et al., 1991; Thompson et al., 1993). These studies showed that the Mediator is essential for TF-dependent activated transcription during *in vitro* assays. Isolation of the complex in mammalian cells followed briefly afterwards and using different approaches several laboratories isolated similar complexes that later were recognized to be identical (Bourbon et al., 2004; Fondell et al., 1996; Gu et al., 1999; Ito et al., 1999; Naar et al., 1999; Rachez et al., 1998; Ryu et al., 1999; Sun et al., 1998).

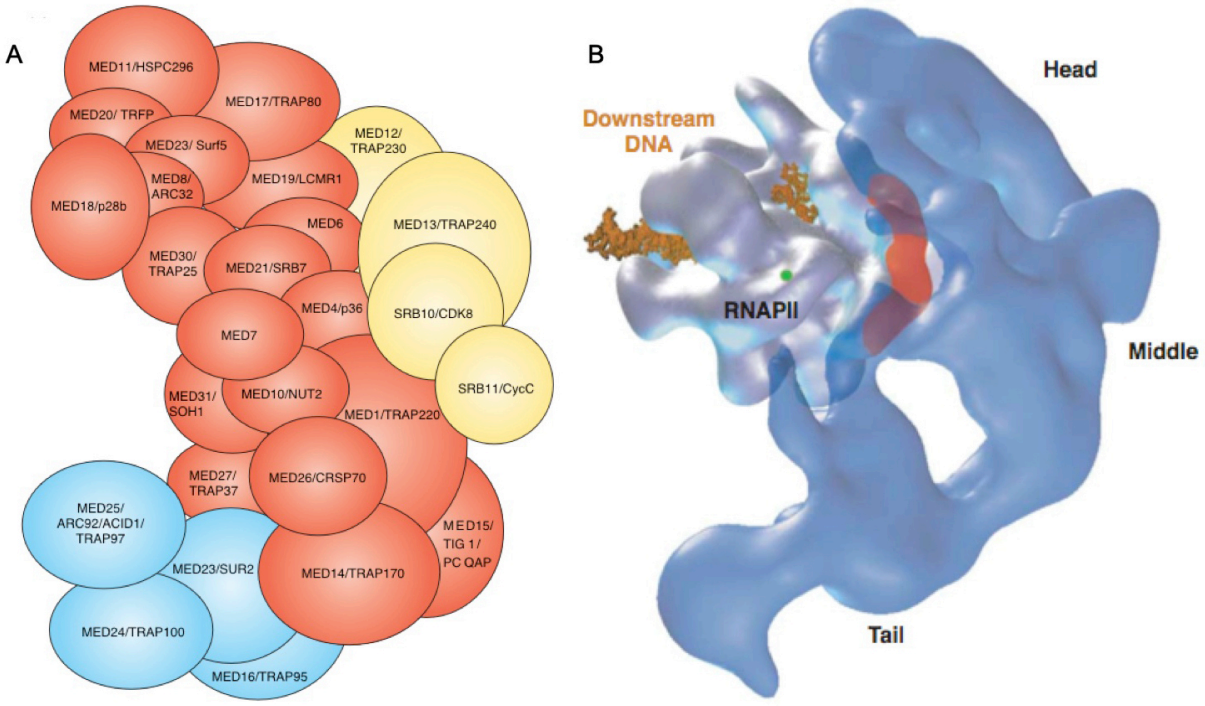

Figure 1.2 The Mediator complex and its interaction with Pol II

(A) Subunit composition of the Mediator complex. In blue, subunits of the tail module; in yellow of the CDK8 module and in red the head and middle modules. Represented are synonyms for the several MED proteins. Figure modified from (Malik and Roeder, 2005). (B) Interaction of Mediator with Pol II. Modeling of the interaction was done by fitting the cryoelectron microscopy structure from Pol II with electron microscopy reconstructions of the Mediator. Figure modified from (Chadick and Asturias, 2005).

The Mediator is a highly conserved structure, present in all eukaryotic organisms. However, conservation among its different subunits is not strong across different species. Some subunits have a very low degree of homology and in some organisms certain subunits are not present (Bourbon, 2008). In mammals the complex currently has 30 identified subunits and an approximate molecular weight of two million daltons (MDa) (Sato et al., 2004). The complex is organized in a modular fashion, containing four modules. The subunits in the head module are able to interact with the Pol II machinery (Takagi et al., 2006). The tail and middle modules contain subunits that are able to bind/interact with transcription factors. The CDK8 module contains a cyclin dependent kinase, which among other roles, is able to phosphorylate Pol II and TFIIH and directly influence their respective functional activities (Figure 1.2). This organization allows the complex to directly convey information from DNA-bound TFs to the transcription machinery (Conaway et al., 2005). The Mediator is not only essential for all activated transcription i.e., transcription enhanced by TFs, but in yeast it has been shown that is also required for the transcription of practically all genes since it also participates in basal, non-activated transcription. In essence, the function of the Mediator is closer to that of a GTF than to other coregulators of transcription (Takagi and Kornberg, 2006).

There are still many open questions concerning the mechanisms of Mediator activity. One of the major bottlenecks in Mediator studies is its big size and multisubunit composition, which renders isolation via recombinant proteins impossible (Taatjes, 2010). Consequently, the Mediator must always be purified from protein cellular extracts and its low expression levels present a challenge. Moreover, it is hard to infer the function of the different subunits by searching for conserved functional protein motifs, as practically none are to be found. A recent analysis of its biochemical composition highlighted however how prone the Mediator is to protein-protein interactions because of its high malleability (Toth-Petroczy et al., 2008).

There are two types of subunits within the Mediator. Some subunits are required for the interaction with specific TFs and therefore have a gene-specific character, as they are required

for the transcription of only a subset of genes, those controlled by the TFs. Others have a broader role and are required for all functions of the complex and therefore necessary for the transcription of all genes.

1.2.1 Mediator control of Pol II and the subunits required for all functions of the complex

If a certain Mediator subunit is responsible for a process that affects all functions of the complex then in a genetic model where this subunit is absent, a generalized defect in transcription should be observed. This hypothesis was tested utilizing several yeast mutants for Mediator subunits. While some mutant strains displayed specific phenotypes, others had a general defect in transcription and were not viable (Myers and Kornberg, 2000). In mammals, using mouse embryonic stem (ES) cells, a similar phenotype was observed when the *Med21* gene was targeted (for deletion), which rendered the cells unviable (Tudor et al., 1999).

Loss of the architectural structure of the complex may also lead to disruption of Mediator function and thus to compromised cell viability. The Mediator in a SRB4 yeast mutant strain dissociates at the Head/Middle boundary and consequently all transcription ceases (Linder et al., 2006). This aspect is conserved in insects, as the Mediator homologue protein in *Drosophila*, MED17, when disrupted also causes a cell viability defect (Boube et al., 2000).

If a mutation affects proteins that are responsible for the interaction and activation of the transcription machinery, this also leads to defects in all functions of the Mediator and consequently compromises cell viability. In mice, loss of Cdk8 leads to early embryonic death prior to uterine implantation, demonstrating the importance of this subunit in generalized transcription and cell survival (Westerling et al., 2007).

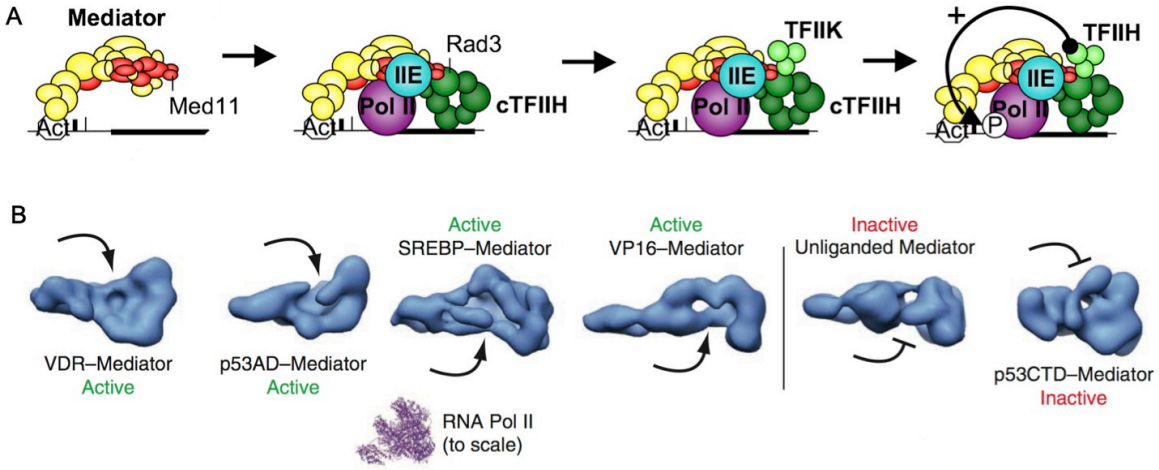

Figure 1.3 How the Mediator controls Pol II activity

(A) Model explaining how the Mediator controls assembly of the PIC. Upon binding to a TF or activator (Act) the Mediator is necessary for Pol II, TFIIE and TFIIH recruitment to the proximal promoter. Essential Mediator subunits are drawn in red, non-essential in yellow. Med11 interacts with the Rad3 subunit of TFIIH and this interaction is required for CTD phosphorylation and consequent activation of Pol II by another TFIIH subunit, TFIIK. Figure modified from (Esnault et al., 2008). (B) Electron microscopy reconstructions of Mediator structure upon binding of different TFs and at an unliganded state. Different interactions lead to different structural shifts. Activators induce formation of a pocket domain where Pol II fits (arrows). Unliganded Mediator or interaction with a non activator (p53CTD) blocks pocket formation and inhibits interaction with Pol II. Figure modified from (Meyer et al., 2010).

Although these genetic studies have helped us to better understand how the Mediator functions, it still remains elusive how the transcriptional machinery is activated via this complex. Several hypotheses have been suggested, including: recruitment of Pol II, assembly of the PIC, and activation of Pol II and GTFs via phosphorylation or via structural shifts within the complex.

In yeast it has been shown that upon TF binding, the Mediator is recruited to the proximal promoter and only then Pol II is recruited (Bhaumik et al., 2004; Cosma et al., 2001). These studies are in line with earlier reports that the Mediator can form a holoenzyme complex with Pol II (Chadick and Asturias, 2005; Kim et al., 1994). Besides Pol II recruitment, the Mediator is responsible for the assembly of GTFs at the promoters of activated genes. TFIIB, TFIID, TFIIE and TFIIH are among the GTFs known to be directly bound and recruited by the Mediator as illustrated in Figure 1.3 (Baek et al., 2006; Esnault et al., 2008). Moreover, it was observed that the Mediator, together with TFIIH and TFIIE, stays on the promoter of genes while Pol II is transcribing potentially to facilitate transcriptional reinitiation (Yudkovsky et al., 2000). Mediator

interaction with TFIIH gains special relevance since TFIIH can phosphorylate the carboxy terminal domain (CTD) of the largest subunit in Pol II. In mammals the CTD tail of Pol II is composed of 52 repeats of the consensus heptapeptide Tyr-Ser-Pro-Thr-Ser-Pro-Ser and phosphorylation of Ser5 is required for promoter escape, whereas phosphorylated Ser2 marks productive elongation (Brookes and Pombo, 2009). Thus, the Mediator controls promoter escape of Pol II since it is necessary for phosphorylation of Ser5 by TFIIH (Sogaard and Svejstrup, 2007).

It has also been suggested that structural shifts within the Mediator can facilitate the control of the activities of the transcriptional machinery. The high malleability of the complex facilitates such a mode of action. Different TFs target different Mediator subunits and each of these interactions can lead to a different structure of the complex (Taatjes et al., 2004b). In agreement with this, a recent study showed that when the activation domain of p53 binds the Mediator it induces the formation of a large pocket domain at the Pol II interaction site, which correlates with the activation of transcription. Pol II CTD tail phosphorylation by TFIIH is also dependent on this structural shift (Figure 1.3) (Meyer et al., 2010). The same laboratory has shown that structural shifts upon TF binding are important for recruitment of other coactivators like CBP or p300 (Ebmeier and Taatjes, 2010).

A recent report suggested yet an additional mechanism by which the Mediator affects transcription and controls gene expression. Kagey and colleagues discovered upon studying genome-wide DNA localization that the Mediator was, as expected from previous reports, found at both the binding sites of TF on distal promoters and at transcription start sites. Cohesin, a protein that can form rings around two DNA segments, is present at the same locations and together with the Mediator is required for the formation of the DNA loop that allows communication between the two DNA regions. Thus it appears that the Mediator connects transcription factors and the Pol II machinery not only by bridging them but also by leading to a physical loop that puts the two regions in closer proximity (Kagey et al., 2010).

1.2.2 The CDK8 module

This Mediator module consists of a cyclin dependent kinase (CDK8), its cyclin pair (CycC) plus Med12 and Med13, two of the complex largest subunits (Blazek et al., 2005). The module interacts transiently with the core Mediator and is mainly associated with transcriptional repression (Casamassimi and Napoli, 2007; Holstege et al., 1998). It was originally proposed that CDK8-bound mediator was unable to bind Pol II (Elmlund et al., 2006) but currently it is thought that other mechanisms are responsible for the observed repressing activity.

In yeast, CDK8 can phosphorylate the CTD tail of Pol II prior to establishment of the PIC, thus inhibiting transcription (Hengartner et al., 1998). In human cells CDK8 is able to repress transcription by phosphorylating TFIIH leading to its inhibition (Akoulitchev et al., 2000). Mechanisms independent of the kinase activity have also been suggested where the module can repress even reinitiation events by changing Mediator structure upon binding (Knuesel et al., 2009a).

In contrast, recent studies have shown that the CDK8 module can exert a positive effect on transcription. CDK8 module containing Mediator is recruited to P53 binding sites on the P21 promoter, and is necessary for gene activation (Donner et al., 2007). CDK8 can also recruit the positive transcription elongation factor b (P-TEFb) which phosphorylates Pol II allowing it to

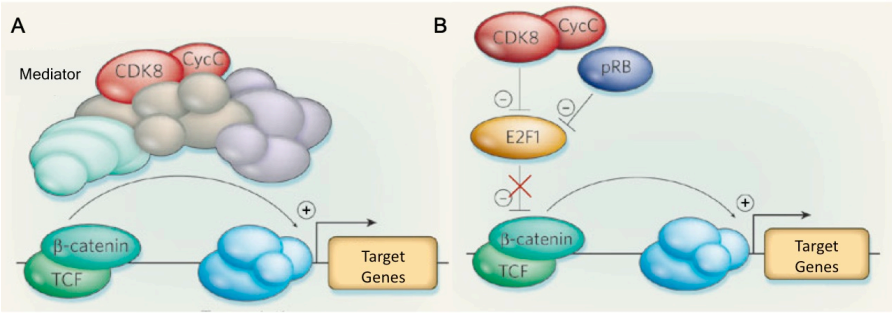

Figure 1.4 Role of CDK8 in colon cancer

CDK8 is an oncogene that acts by two different mechanisms. (A) CDK8 is a coactivator for the TCF/β-catenin complex whose target genes lead colon cancer initiation. (B) CDK8 also suppresses E2F1 inhibition of the TCF/β-catenin complex thus protecting its pernicious transcriptional activity. Figure modified from (Bernards, 2008).

elongate (Donner et al., 2010). In a more indirect fashion, CDK8 containing Mediator can recruit GCN5L which together with CDK8 can phosphorylate serine 10 in histone 3 leading to the activation of transcription (Meyer et al., 2008). Localization of the CDK8 module on DNA has been studied in genome-wide analyses and showed that the CDK8 containing Mediator binds to the same promoters as the core complex regardless of their transcriptional status. This also indicates that the CDK8 module can be involved in transcriptional activation (Andrau et al., 2006; Zhu et al., 2006).

Finally, the CDK8 subunit has major clinical relevance. It can act as an oncogene by influencing the activity of Wnt signaling during colon cancer development (Firestein et al., 2008). This signaling pathway (described in more detailed below) is necessary for colon cancer initiation and Cdk8 influences the activity of its downstream effector, β-catenin. Moreover, CDK8 protects the activity of β-catenin by targeting and inhibiting the action of E2F1, an inhibitor of β-catenin that acts as a tumor suppressor (Figure 1.4) (Morris et al., 2008).

1.2.3 Mediator subunits with gene-specific functions

By binding to TFs via its gene-specific subunits the Mediator acts as a control panel where the different signaling pathways converge, allowing the cell to sense the stimuli to which it is exposed (Table 1.1). It was due to its gene-specific functions that the first mammalian Mediator subunit was identified and the complex isolated. Roeder and colleagues established the Mediator as a coactivator required for thyroid receptor (TR)-activated transcription (Fondell et al., 1996). The thyroid hormone receptor is a TF that belongs to the nuclear receptor (NR) superfamily of TFs and other NRs like the vitamin D receptor (VDR) or the peroxisome proliferator-activated receptor (PPAR) also use the MED1 (known also as TRAP220) subunit to recruit the Mediator to the promoter of their target genes. This recruitment is necessary for the multitude of processes controlled by NRs during development, homeostasis and disease (Ge et al., 2002; Ito et al., 2000; Rachez et al., 1999; Rachez et al., 1998; Vijayvargia et al., 2007).

Table 1.1 Known interactions between TFs and Mediator subunits

Viral Activators	Mediator Subunits	TFs	Mediator Subunits	TFs	Mediator Subunits
VP16	15-17-25	CEBP	1-23	SREBP	14-15
E1A	23	ELF3	23	p53	17
RTA	12	ELK1	23	Sox9	12
		PGC1-α	1-16-17	Gal4	15
		NRs	1	Gcn4	2-3-15-16-22
		Smad2-4	15	Tup1	21
		GLi3	12	p65(NfκB)	17

NRs are activated by lipophilic compounds such as hormones and therefore are able to diffuse to the nucleus without specific membrane receptors or other intricate mechanisms as it is often found in all other eukaryotic signaling pathways. Because of this simplicity, NRs are a traditional model to study eukaryotic transcription (Belakavadi and Fondell, 2006). Important mechanisms concerning Mediator gene-specific functions have been elucidated using the MED1-NRs interaction as a model. Among these, it was shown that MED1 can, upon PPAR activation, recruit both p300 and PGC-1α, two coactivators that lead to chromatin modification and transcriptional activation of PPAR targets (Wallberg et al., 2003). CCAR1 is a coactivator that controls cell-cycle and apoptosis and was shown to be necessary for MED1 recruitment to the NRs (Kim et al., 2008). Finally, MED1-NRs studies described a mechanism where Mediator subunits associate with the complex only upon stimulation of the transcription factor they bind to (Belakavadi et al., 2008).

Studies in human cells allowed identification of other pairs of interacting Mediator subunits and TFs, including MED17 and p65 (van Essen et al., 2009), SREBP with MED15 (Yang et al., 2006) and of the viral activator VP16 with both MED17 and MED25 (Mittler et al., 2003). Studies in *Xenopus laevis* showed that smad2 and smad4 of the TGFβ/Activin/Nodal signaling pathway bind med15 for recruitment of the Mediator to the promoter of its target genes (Kato et al., 2002). Using mouse models generated by gene-targeting in ES cells, the following subunits have been shown to possess gene-specific functions: Med24 (Ito et al., 2002), Med31 (Risley et al., 2010) and Med23 (Stevens et al., 2002). Med23 has recently been shown to be necessary for transcription mediated by the following TFs: Elk1, Elf3, E1A and the Cebp (Wang et al., 2005).

Plants do not have NRs, and interestingly their Mediator also lacks a MED1 homologue, which once more highlights the gene-specific function of this subunit. Moreover it exemplifies how the Mediator composition was subject to evolution and how it helps organisms to better adapt to their environment (Bourbon, 2008).

1.3 The Mediator subunit MED12

MED12 is an interesting subunit as it has been suggested that it participates in many of the general functions of the Mediator, but also in several gene-specific processes. Its function and role within the Mediator remain a mystery, but many reports suggest that it is essential for the orchestration of gene expression by the complex. MED12 is found in animals, fungi and plants and has a strong sequence-conservation between humans and primates (99%) and humans and mice (96%) (Bourbon, 2008).

In mammals, *MED12* is located on the X chromosome, and both in humans and mice consists of 45 exons producing a 7 kb mRNA. MED12 has more than 2000 amino acids and it is organized in four domains based on its structure and sequence identity. The leucine and leucine-serine-rich domains cover most of the protein. Additionally, it has a proline-glutamine-leucine-rich domain and, at the C-terminus of MED12, a motif called the OPA domain because of its strong sequence identity with the *Drosophila melanogaster* Opa (odd-paired) motif (Figure 1.5) (Philibert and Madan, 2007).

Figure 1.5 Structure of the human MED12

Represented are the four MED12 domains according to biochemical composition. Arrows indicate sites of mutations known to cause the Opitz and Lujan syndrome as well as the location of the HOPA12^{bp} polymorphism (see section 1.3.3).

1.3.1 MED12 and its general role in transcription

MED12 is part of the CDK8 module and therefore is involved in many of the processes that are controlled by CDK8 and its partners. Its precise role within the Mediator is however not yet clear. As with the other components of the module it was originally identified as a negative

regulator of transcription since yeast deficient for MED12, showed derepression of most genes (Carlson, 1997).

Two recent biochemical studies have helped to elucidate the role of MED12 within this module and ultimately during transcription. The kinase activity of CDK8 requires MED12 to phosphorylate Pol II, TFIIH and histones, which is crucial for positive and negative regulation of transcription (Knuesel et al., 2009b). Moreover, for CDK8 module-dependent transcriptional repression, the kinase activity of CDK8 is not necessary but MED12 and MED13 must be present in the complex and act as a molecular switch that changes the Mediator structure to repress reinitiation of transcriptional events (Knuesel et al., 2009a).

1.3.2 Gene-specific functions of MED12

In contrast with the few studies concerning the influence of MED12 in the general functioning of the Mediator, a good number of reports, many in eukaryotic organisms, have suggested several gene-specific roles for MED12. In *Arabidopsis thaliana*, MED12 together with MED13 is required for embryo patterning and temporal growth coordination (Gillmor et al., 2010). In *Caenorhabditis elegans*, MED12 is necessary for asymmetric cell division, Wnt signaling-regulated cell fusion (Yoda et al., 2005; Zhang and Emmons, 2000), and RAS-dependent vulval fate specification inhibition (Moghal and Sternberg, 2003). Eye development in *Drosophila melanogaster* relies on MED12, which serves as a coactivator for the transcription factor, Atonal (Lim et al., 2007; Treisman, 2001). Also in *Drosophila*, MED12 is necessary for correct output of developmental stimuli from the Wnt signaling pathway by serving as a coactivator for its transcriptional effectors (Carrera et al., 2008). The Wnt signaling pathway was first discovered in *Drosophila* and later homologs of its components were found in practically all animals where it acts as an essential coordinator of developmental, and several physiological, processes (Clevers, 2006). The importance of MED12 for Wnt signaling seen in invertebrates seems to be conserved in vertebrates since the human MED12 can bind β-catenin, which is the downstream

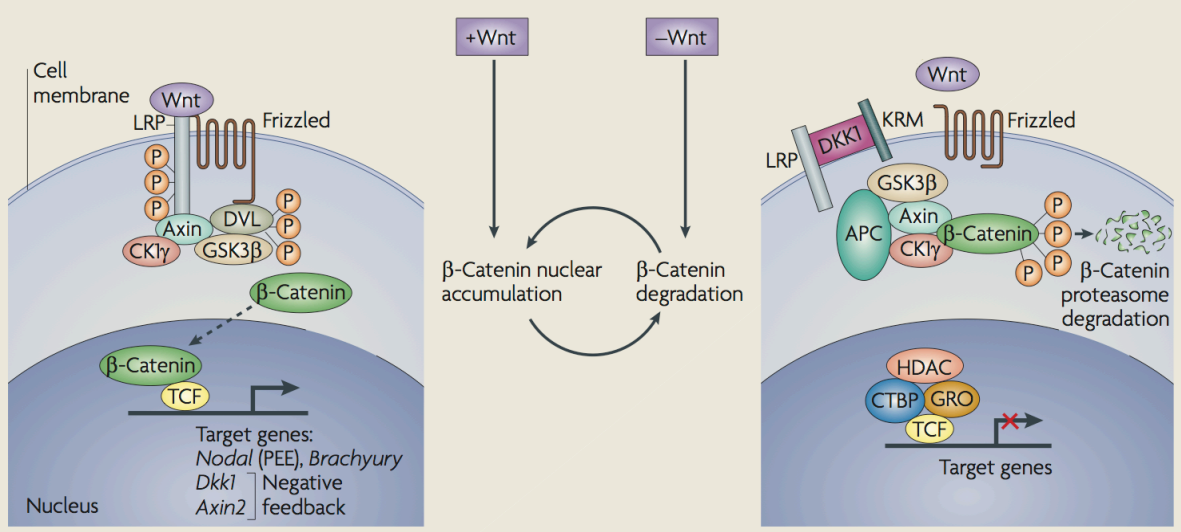

Figure 1.6 The canonical Wnt/β-catenin pathway

In the absence of Wnt ligands (right) β-catenin is targeted for phosphorylation by the destruction complex, composed of APC, GSK3β and Axin, which promotes its degradation. TCF target genes remain repressed due to the action of corepressors such as HDACs and groucho (GRO). In the presence of Wnt ligands (left) the LRP receptors are phosphorylated and recruit the disheveled (DVL) and Axin proteins. This allows β-catenin to translocate to the nucleus where together with TCF it can activate transcription of its target genes. Figure modified from (Arnold and Robertson, 2009).

effector of the Wnt signaling pathway (Figure 1.6). Additionally, MED12 is necessary for the activation of β-catenin responsive promoters driving luciferase reporter expression (Kim et al., 2006). Other interactors of human MED12 include GLI3, a downstream target of the sonic hedgehog (Shh) signaling pathway, which recruits the Mediator via MED12 to repress its target genes (Zhou et al., 2006). Additionally, MED12 interacts with G9a, a histone methyl transferase that is responsible for transcriptionally repressive histone 3 residue K9 mono- and di-methylation, thus suggesting one more mechanism by which MED12 can negatively regulate transcription (Ding et al., 2008).

Study of the zebrafish *Danio rerio* has allowed the discovery of several developmental processes that require MED12 in vertebrates. Various *med12* zebrafish mutants have been described, showing defects in neural crest formation, chondrogenesis and organogenesis of brain, liver, pancreas and kidney (Hong et al., 2005; Rau et al., 2006; Shin et al., 2008; Wang et al., 2006). Some of these phenotypes can be explained by the failure of target gene activation by

transcription factors such as Sox9, Sox32 and Foxa2. The role of MED12 as a coactivator for SOX9 has also been shown in human cells (Zhou et al., 2002).

Recently it was suggested that Med12 is necessary for pluripotency of murine ES cells (Tutter et al., 2008). It is known that the transcription factors Nanog, Oct4 and Sox2 are responsible for maintenance of ES cells pluripotency and repression of differentiation. According to this recent report, Med12 can bind Nanog and is involved in the regulation of *Nanog* itself, and Nanog target genes therefore playing a role in the regulatory core circuit that maintains ES cells pluripotent (Tutter et al., 2008).

1.3.3 Mutations in MED12 that cause human diseases

Two human X-linked mental retardation (XLMR) syndromes have been associated with missense mutations in *MED12*. Both mutations cause single amino acid substitutions and are located in the Leucine-Serine rich domain separated by only a few amino acids. The Opitz-Kaveggia syndrome is caused by an arginine to tryptophan substitution (Risheg et al., 2007) while a serine to asparagine mutation causes the Lujan syndrome (Figure 1.5) (Schwartz et al., 2007).

The symptoms patients develop confirm the role of MED12 in gene specific functions and relate with phenotypes observed in zebrafish mutants such as the defects in neural crest formation which can lead to cranial-facial dysmorphia as seen in Lujan syndrome patients (Schwartz et al., 2007). The imperforate anus in Opitz-Kaveggia patients is a defect that can be related with the endoderm phenotypes also observed in zebrafish mutants (Risheg et al., 2007). Overlapping symptoms of both syndromes include mental retardation and macrocephaly. Additionally, the association of MED12 with these two XLMR syndromes suggests a role during neurological development for which the leucine-serine rich domain appears to be important (Figure 1.7).

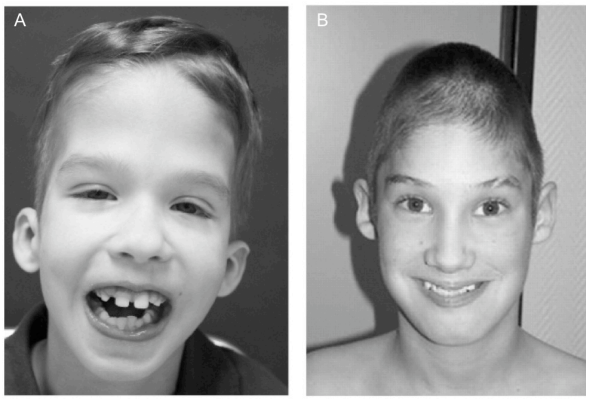

Figure 1.7 The Opitz-Kaveggia and Lujan Syndromes.

(A) Opitz-Kaveggia patient at age 7 with small ears, tall and prominent forehead, and frontal hair upsweep. Figure modified from (Lyons et al., 2009) (B) Lujan syndrome patient at age 11 displays scaphocephaly, upslanting palpebral fissures, short philtrum and micrognathia. Figure modified from (Schwartz et al., 2007)

An insertional polymorphism in the OPA domain of MED12, known as HOPA^{12bp} has been associated with a modest risk (1.5%) for a behavioral phenotype including psychosis and schizophrenia. Although in some populations this association failed to be proven significant, this study highlights the importance of MED12 for XLMR disorders (Philibert and Madan, 2007).

1.4 Studying eukaryotic transcription with mouse models and aim of this thesis

The mouse has been an excellent model for vertebrate genetics since the beginning of the last century. Its fast life cycle (3 weeks gestation, sexual maturity at 6 weeks), easy husbandry, availability of inbred strains, and similar anatomy and physiology with humans has made it essential for biomedical and developmental studies. Classical forward genetics experiments with induction of random mutations provided excellent genetic models that, together with the first transgenic mice derived by pronuclear injection of fertilized oocytes, were instrumental in developmental disease and physiological events (Brinster et al., 1981; Harbers et al., 1981; Paigen, 2003a). A major advance in mouse genetics was the establishment of culture conditions for pluripotent embryonic stem cells that can be genetically modified and then be used to derive mice carrying different mutations (Evans and Kaufman, 1981; Martin, 1981). This allowed the generation of “knockout models” where the function of a gene can be studied *in vivo* by disrupting its coding sequence, and also of mice carrying other modifications such as specific point mutations (Smithies et al., 1985; Thomas and Capecchi, 1987).

The next revolution in the field was the introduction of the Cre-*loxP* system into mouse genetics. Cre is a site-specific recombinase of the bacteriophage P1 that recognizes the 34 bp *loxP* sequence. If two *loxP* sites have the same orientation, Cre-mediated recombination will lead to excision of the DNA they flank (Sauer and Henderson, 1988). For the targeting of numerous genes in ES cells, *loxP* sites have been introduced to flank whole genes or critical exons. Mice derived from these ES cells express the targeted gene(s) at wild-type levels since the 34 bp *loxP* sequences normally do not interfere with gene expression (Paigen, 2003b). When mating such mice with mouse lines expressing Cre recombinase, the progeny containing both transgenic alleles will recombine at the *loxP* sites resulting in the excision of the flanked DNA fragment (Schwenk et al., 1995). Mice for which Cre expression is driven by tissue-specific

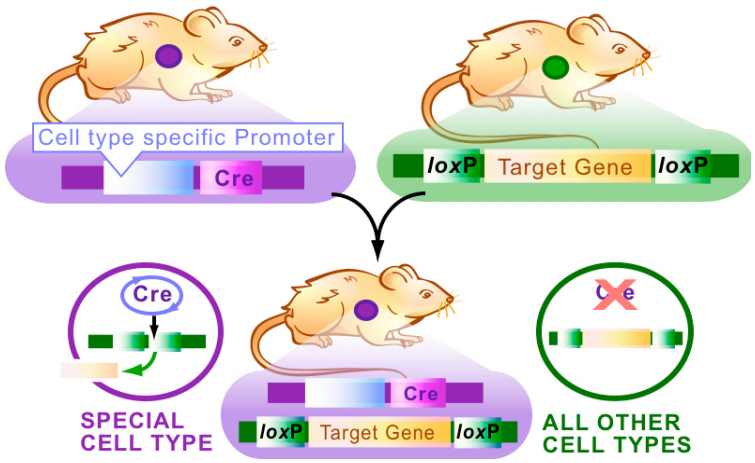

Figure 1.8 The Cre *loxP* system

Mating of mice expressing Cre through a tissue-specific promoter with mice with the target gene flanked by *loxP* sites results in gene excision only in cells of the progeny where the tissue-specific promoter is active. All other tissues express the target gene normally. Figure from (Pechisker, 2004).

promoters or that are inducible, permit fine control of the excision event (Figure 1.8) (Danielian et al., 1998).

The biological advantages of the mouse combined with the ability to generate genetically modified animals have made an impact across all fields of biomedicine. Mouse models have contributed to the study of fundamental biochemical processes such as eukaryotic transcription. “Knockout” mice of DNA methyltransferases are a good example of this as their physiological role was unveiled through gene-targeting of mouse ES cells (Okano et al., 1999). Concerning the Mediator, mouse models have helped to reveal the roles and mode of action of several subunits (Ito et al., 2002; Ito et al., 2000).

The aim of this thesis was to generate mice carrying mutant alleles of *Med12* by using the Cre-*loxP* system to target the gene in ES cells. *Med12* expression can then be modulated according to the Cre mouse lines used to achieve excision. Med12 was chosen among the other Mediator subunits because of the hitherto lack of a mouse model and the many reports suggesting important developmental functions of this gene in several other organisms. These experiments can clarify the elusive role of Med12 and investigate if the described gene-specific functions are conserved in mammals, or if on the other hand, Med12 functions in a more general fashion within the Mediator.

2 Publication 1 - Med12 is essential for early mouse development and for canonical Wnt and Wnt/PCP signaling

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2.1 Experimental Contributions

Manuela Scholze performed most of the whole-mount *in situ* hybridizations.

Wilfrid Bleiss did the scanning electron microscopy experiments.

Heinrich Schrewe supervised and helped planning the experiments.

All other experiments were done by me including: all cell culture work; design and cloning of the targeting construct; manipulation and screening of mouse ES cells; RNA, protein and histological analyses and immunostainings.

2.2 Genetic models of *Med12* used in the publication

This study describes a classical gene-targeting experiment via homologous recombination in mouse ES cells, and the analysis of the developmental phenotypes seen in embryos generated from different mutant alleles. A *Med12* floxed (flanked by *loxP* sites) allele was generated and named $Med12^{flox}$. ES cells containing this allele expressed Med12 at wild-type levels, as the *loxP* sites did not interfere with transcription of the targeted gene. Recombination between the *loxP* sites in $Med12^{flox}$ ES cells catalyzed by Cre recombinase led to excision of the first seven exons. Upon recombination, cells produced no Med12 protein and were referred to as $Med12^{\Delta 1\text{-}7}$. Med12 hypomorphic cells ($Med12^{hypo}$) were fortuitously generated during the process of gene-targeting and expressed only very reduced levels of Med12. Schemes of the different alleles and ES cells characterization can be seen in figures 1, S2 and S7 of the publication and below, in Figure 2.1.

For the studies described here $Med12^{\Delta 1\text{-}7}$, $Med12^{hypo}$, and as a control, $Med12^{flox}$ ES cells, were aggregated with tetraploid wild-type morula-staged embryos. Tetraploid cells can only contribute to extra-embryonic tissues and therefore the generated embryos were fully derived from either control or Med12 deficient cells (Eakin and Hadjantonakis, 2006). This allowed a

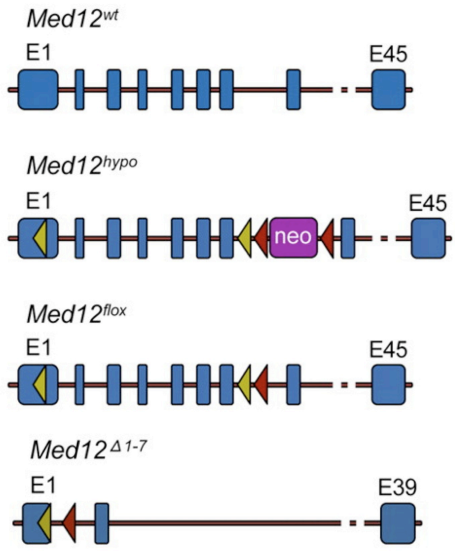

Figure 2.1 *Med12* alleles

Med12 alleles used in this study. $Med12^{hypo}$ ES cells were generated by targeting $Med12^{wt}$ ES cells. The neomycin resistance cassette (neo, in pink) used for transgene integration selection caused a negative interference with *Med12* expression. The first seven exons were flanked by *loxP* sites (green arrowheads). $Med12^{flox}$ cells were generated from $Med12^{hypo}$ ES cells by transient transfection of a plasmid expressing Flp recombinase that recombines DNA at *frt* sites (red arrowheads) and removed the neomycin cassette restoring *Med12* expression. $Med12^{flox}$ ES cells were then transiently transfected with a Cre recombinase expressing plasmid that excised the first seven exons creating the $Med12^{\Delta 1\text{-}7}$ allele.

direct analysis of the defects caused by lack of Med12 during mouse embryonic development. Finally, demonstrating the conditional-null nature of the *Med12*^{flox} allele, the article described the establishment of the *Med12*^{flox} mouse line, which had no obvious phenotype and was fertile.

2.3 Results from publication 1

2.3.1 Gastrulation defects of Med12 deficient embryos

During the sixth day of mouse development the embryonic anterior-posterior axis is established. This occurs through processes of cell differentiation, migration, and embryonic patterning. At the anterior-most tip of the embryo the anterior visceral endoderm (AVE) region is formed after migration of the distal visceral endoderm (DVE) cells from the distal-most tip of the embryo. Then, cells of the epiblast, located at the proximal side of the embryo opposite to the AVE, form the primitive streak and establish the posterior side of the embryo. Here, cells ingress through the primitive streak (PS), after undergoing epithelial-to-mesenchymal transition (EMT) and form the mesoderm (Figure 2.2) (Arnold and Robertson, 2009).

Experiments with *Med12*$^{\Delta 1\text{-}7}$ embryos established Med12 as essential for AVE specification and mesoderm formation through EMT. In the absence of Med12, mouse embryos failed to complete gastrulation and died at embryonic day (E) 7.5. Immunofluorescence and RNA whole mount *in situ* hybridization (WISH) studies using marker genes for both the AVE and PS showed that these structures are not correctly formed in the absence of Med12. AVE marker genes were either not expressed and cells failed to migrate from the distal tip of the embryo. Histological

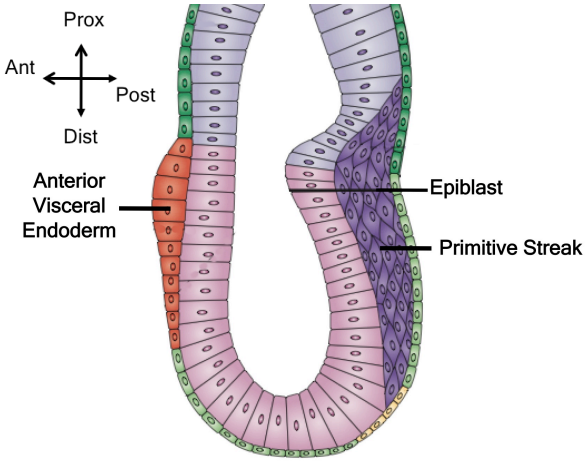

Figure 2.2 Mouse embryo at E7

The scheme represents a sagittal section through a mouse embryo at 7 days of development. Labeled are tissues mentioned in the text. Additionally, the following tissues are represented; extraembryonic visceral endoderm – green at the top; extraembryonic ectoderm - light blue; embryonic visceral endoderm – green at the bottom; definitive endoderm – yellow. Figure modified from (Arnold and Robertson, 2009).

analyses suggested that the PS is apparently formed in *Med12*$^{\Delta 1\text{-}7}$ embryos but marker genes of this structure are not expressed and E-cadherin staining demonstrated that the ingressing cells failed to perform EMT and retained epithelial nature. These experiments are described in figure 5 of the publication.

2.3.2 Phenotypes of Med12 hypomorphic embryos at E9.5

As mentioned previously, embryos generated from *Med12*^{hypo} ES cells expressed very limited levels of Med12, up to 10% in comparison to controls. However, this small amount allowed embryos generated from *Med12*^{hypo} ES cells to escape early lethality at the gastrulation stage and died at E10.5. The experiments in figures 1, 2 and S4 of the article report the developmental defects of *Med12*^{hypo} embryos and an example can be seen in Figure 2.3.

The most likely cause of death of *Med12*^{hypo} embryos was a defective cardiovascular system. Med12-deficient embryos had an enlarged heart that was not properly differentiated and did not form the appropriate loops characteristic of this stage that shape the final structure of the organ. The most obvious defect seen in mutant embryos was a failure of neural tube closure, which was fully penetrant. All *Med12*^{hypo} embryos did not achieve closure in the head and in most

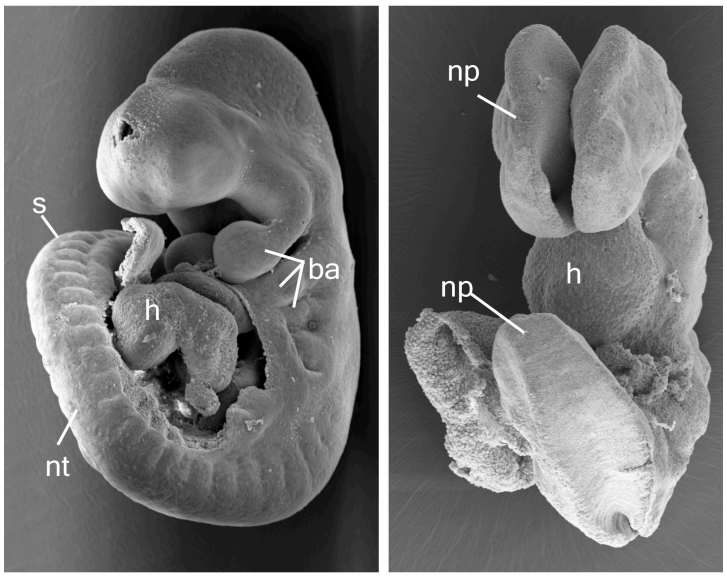

Figure 2.3 *Med12*^{hypo} embryos survive up to midgestation

Embryos derived from *Med12*^{flox} (left) or *Med12*^{hypo} (right) ES cells at E9.5 visualized by scanning electron microscopy. The heart (h) in mutants was enlarged, poorly differentiated and stayed at the midline. Branchial arches (ba) were not present and only a few small and irregular somites (s) were formed. The neural tube (nt) did not close in the caudal and rostral region. At the posterior most end, the neural plate (np) failed to elevate and remained flat.

of the caudal region. An important pool of pluripotent undifferentiated cells resides in the folds of the neural plate. These cells, called neural crest cells (NCCs), begin migrating during neural tube closure start and contribute to the formation of the branchial arches, which later are incorporated into many structures of the skull and build-up the peripheral nervous system. In *Med12* mutants branchial arches were absent or dramatically reduced.

In addition, *Med12*^{hypo} embryos had a truncated body axis that did not fully elongate posteriorly. This was caused by a rudimentary unsegmented caudal mesenchyme that failed to express critical marker genes known to regulate its function such as *Tbx6, T* and *Cyp26a1*. Segmentation of the mesenchyme into somites, a process known as somitogenesis, also occurred defectively. Somites are transient epithelial structures that contribute to vertebrae and skeletal musculature formation. Reduced Med12 levels caused formation of smaller, irregular and undifferentiated somites.

2.3.3 Med12 is an *in vivo* coactivator of β-catenin

In human cells Med12 has been shown to bind β-catenin and to activate a luciferase reporter with a β-catenin responsive promoter (Kim et al., 2006). As represented in Figure 1.6, β-catenin is the downstream effector of canonical Wnt signaling, which is one of the most important signaling pathways regulating organism development and homeostasis, and also several disease events. Examples of this include the known roles of canonical Wnt signaling during embryonic patterning and growth, organogenesis, and cancer (Grigoryan et al., 2008). *Med12* mutant embryos identified Med12 as a coactivator for β-catenin during early mouse development.

The failure in anterior migration of the visceral endoderm and the lack of a functional primitive streak in *Med12*$^{\Delta 1\text{-}7}$ embryos are classical examples of defective Wnt signaling. Moreover, expression of brachyury (T), one of the best characterized direct targets of canonical

Wnt signaling during PS formation, was completely abrogated at both the RNA and protein levels. These results are depicted in figure 5 of the article.

Similarly, figures 2 and 4 of this Publication show experiments with *Med12*^{hypo} embryos that revealed additional Wnt-regulated processes dependent on Med12 such as axis elongation and somitogenesis . Elongation of the body axis is accomplished through proliferation of the caudal end mesenchyme (Wilson et al., 2009). Expression of several marker genes in this tissue that are important for its function, are controlled by canonical Wnt signaling and was defective in *Med12* mutants. Somitogenesis is a tightly regulated process where several signaling pathways, including Wnt, fibroblast growth factor (Fgf), and Notch, regulate the precise timing for a new somite pair to be formed as the body axis extends (Aulehla and Herrmann, 2004). This is accomplished through oscillatory expression of the gene network components and in *Med12*^{hypo} mutants, like in many Wnt-defective mouse models, oscillation is lost resulting in perturbed somitogenesis. Finally, as in the complete null embryos, *Med12*^{hypo} mutants had a marked decrease in expression of several genes known to be direct targets of canonical Wnt/β-catenin signaling such as *Axin2, Ccnd1, Myc* and *Dkk1*.

2.3.4 Med12, neural tube closure and planar cell polarity

During murine development, closure of the neural tube starts at E8.5, when the embryo has seven pairs of somites. It starts at three different closure points and progresses in a bidirectional fashion until the whole axis is fully closed. By E9.5 the process is complete, except at the caudal neuropore. As the posterior axis elongates closure at this site occurs continuously. Neural tube closure requires tight control over several processes such as cell division, intercellular interactions, migration, patterning, and changes in morphology. This is necessary to control the precise timing and location for the neural plate to elevate and then, when the neural folds come within close proximity, to allow fusion at the midline (Figure 2.4) (Copp and Greene, 2009; Copp et al., 2003).

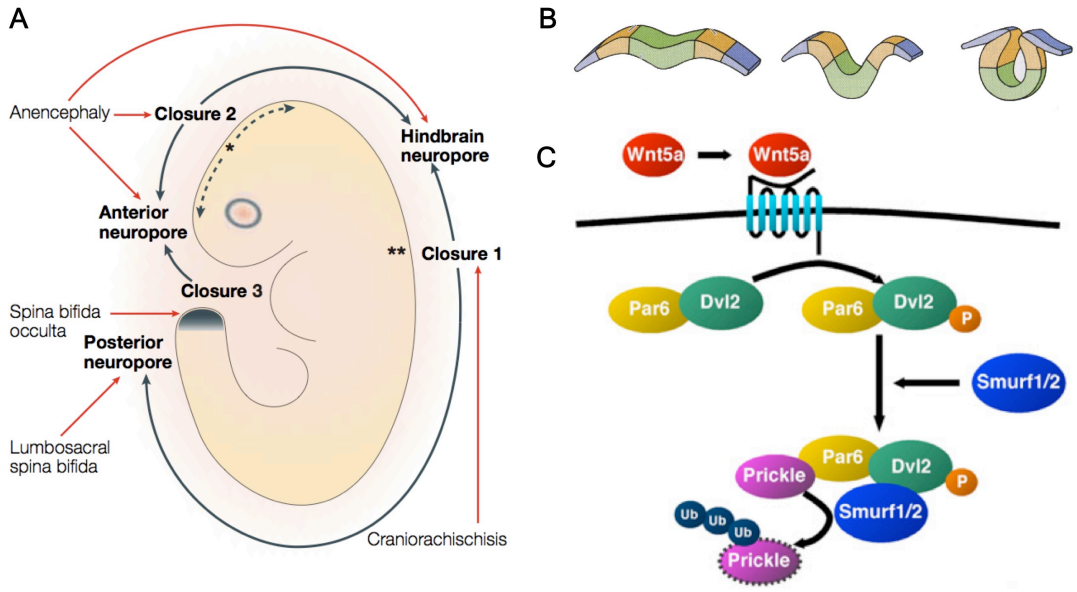

Figure 2.4 Neural tube closure and the Wnt/PCP pathway

(A) Initial points of neural tube closure in the mouse embryo. Black arrows indicate closure directions followed after initial contact. Diseases caused by failure of neural tube closure are also shown and their designation depends on the site of failure, which is represented in red. This will be mentioned in section 3.3.3. Figure from (Copp et al., 2003). (B) Representation of the process of neural tube closure. Neural folds elevate, become apposed at the midline, fuse and then the neural tube is covered by epithelial ectoderm (in blue). Figure modified from (Gilbert, 2000). (C) Model how Prickle1 degradation and consequently localization is controlled by binding of the non-canonical Wnt5 ligand to Frizzled. Upon binding, the activated Par6/Dvl2 complex recruits the Smurf1/2 ubiquitin ligase that degrades Prickle1 only on one side of the cell conferring its asymmetric localization. Figure from (Narimatsu et al., 2009).

As mentioned previously, all *Med12*^{hypo} embryos failed neural tube closure in the head (closure points 2 and 3) and at the caudal most end (closure point 1 at posterior end) of the body axis. Although most of the analyzed embryos achieved closure in a segment of the spinal area (initial closure point 1), a small proportion (15%) had no closure and presented a fully open neural plate, as represented in figure S6 of the publication. Interestingly, out of over 150 genes known to interfere with neural tube closure, all those that cause such a striking phenotype belong to the same developmental signaling pathway, the non-canonical Wnt-planar cell polarity (PCP) pathway (Harris and Juriloff, 2010). This suggested that, in Med12 mutants, Wnt/PCP signaling might be deregulated and that Med12 may play a role establishing this pathway.

The Wnt/PCP signaling pathway, like its canonical counterpart, functions via Wnt ligands that bind frizzled receptors and activate the protein disheveled (Dvl). In PCP, however, Dvl activation will influence the cellular localization of downstream proteins like Prickle1, Vangl2, and

Par6. Proper localization of these proteins is required for cells to establish polarity within an epithelial plane as occurs with the hairs of the *Drosophila* wing. In vertebrates PCP has been implicated in the regulation of convergent extension (CE), neural tube closure, eyelid closure, and hair bundle orientation in sensory cells of the inner ear (Figure 2.4) (Wang and Nathans, 2007).

In *Med12*^{hypo} embryos, Prickle1 protein, instead of being localized at the membrane of neural plate cells was found dispersed through the cytoplasm (figure 3 of publication). This has also been shown to occur in mutants for Smurf, an ubiquitin ligase, where mislocalization led to a neural tube phenotype similar to the one observed in *Med12*^{hypo} mutants (Narimatsu et al., 2009). The neural tube closure phenotype along with Prickle1 mislocalization link, for the first time, a Mediator subunit with establishment of the Wnt/PCP signaling pathway.

2.4 Discussion of publication 1

This publication described the generation of a mouse model for the loss of Mediator subunit Med12 that was used to elucidate whether this subunit has gene-specific functions within the complex, or if it is more generally required for all activated transcription. Several reports had already provided supporting evidence for both arguments. The strength of a knockout mouse model resides not only in its high robustness and reproducibility, but also in the *in vivo* validity of the conclusions that can be drawn from studying it.

Generating embryos from two different ES cell lines that either produce no detectable Med12 (*Med12*$^{\Delta 1\text{-}7}$) or have strongly reduced protein levels (*Med12*^{hypo}), has clearly shown that Med12 has gene-specific functions in the Mediator complex. In both types of *Med12* mutants, only specific developmental processes such as gastrulation, heart formation, somitogenesis, or neural tube closure were affected while several other embryonic structures were formed normally. As for the developmental gene-specific functions discovered for Med12, its ability to act as a coactivator for both canonical Wnt and Wnt/PCP signaling provides a significant advance in understanding the biology of the Mediator and how signaling pathways integrate at the level of transcriptional control during mouse development.

Several experiments with *Med12*$^{\Delta 1\text{-}7}$ and *Med12*^{hypo} embryos have clearly shown that canonical Wnt signaling, which is instrumental for many stages of mouse development, requires Med12. This conclusion is further supported by previous results with *Drosophila melanogaster* and *Caenorhabditis elegans* where Med12 has also been linked to Wnt signaling (Carrera et al., 2008; Yoda et al., 2005; Zhang and Emmons, 2000). The previously reported *in vitro* observations that Med12 binds β-catenin and is required for activation of artificial reporter constructs are extended by the experiments described here, and show that these interactions also occur *in vivo* during mouse development and are functionally relevant for activation of Wnt target genes.

The link between Med12 and non-canonical Wnt/PCP signaling is completely novel. Loss of asymmetric Prickle1 at the cellular membrane in *Med12*^{hypo} embryos led to defects of neural tube closure, but the cause of the mislocalization is still unknown. It is possible that Med12 acts as a coregulator for transcription factors regulating core PCP components which control localization of Prickle1, such as Smurf1/2, Par6, or Dvl2. Med12 could also have a more general role in the signaling pathway by serving as a coregulator for TFs acting downstream of the signaling pathway.

It is clear that Med12 is not essential for all functions of the Mediator since several developmental processes and signaling pathways are unperturbed in mutant embryos. It cannot be said, however, that Med12 has no influence on the more general roles of the Mediator since redundancy between the different subunits may exist. In its absence, the potential functions of Med12 during transcriptional activation and repression could be performed by Med13, its module partner.

Finally, the publication described the establishment of the conditional null allele *Med12*^{flox}. Both ES cells and mouse strains with this allele can be considered wild-type as Med12 expression is not affected and no phenotypes were detected. Cre-mediated excision of the first seven Med12 exons results in an effective null-allele where no Med12 protein is produced. This tool is invaluable for additional studies concerning Med12 functions during mouse development and also at later adult stages using conditional Cre-deleter mouse lines.

3 Publication 2 - Mosaic expression of Med12 in female mice leads to exencephaly, spina bifida, and craniorachischisis

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3.1 Experimental contributions

Wilfrid Bleiss did the scanning electron microscopy experiments.

Heinrich Schrewe supervised and helped planning the experiments.

All other experiments were done by me, including planning and supervision of mouse matings, genotyping, protein characterization, phenotypic analyses and skeletal stainings.

3.2 Genetic models of *Med12* used in the publication

This publication describes the first use of the *Med12*^{flox} mouse line, which has no phenotypic abnormality and is fertile. Male *Med12*^{flox} mice were mated with *CMV-Cre* females, the first and best-characterized general Cre-deleter mouse strain wherein the potent cytomegalovirus (CMV) promoter drives transgenic expression of Cre recombinase. Upon mating with mice containing floxed alleles, *CMV-Cre* has been show to promote recombination between *loxP* sites in all cells of the progeny (Schwenk et al., 1995).

For this study, homozygous *CMV-Cre* females were mated with hemizygous *Med12*^{flox} males (*Med12*$^{flox/Y}$). Since *Med12* is located on the X-chromosome, only two genotypes were expected in the progeny. All male embryos received the X-chromosome from their mothers, with a *Med12* wild-type allele and the *CMV-Cre* transgene. They were designated as wild-type controls and represented as *Med12*$^{wt/Y}$*;CMV-Cre*. Female embryos invariably received maternally a wild-type *Med12* allele together with a copy of the *CMV-Cre* transgene and a floxed *Med12* allele from their fathers. These females were named *Med12* heterozygous females (since one of the *Med12* alleles suffered Cre-mediated recombination) and represented as *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre*. Figure 3.1 is a representation of the breeding scheme.

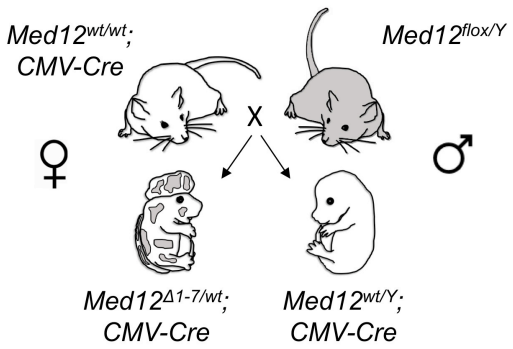

Figure 3.1 Breeding scheme for *Med12* heterozygous females

Mating CMV-Cre homozygous females with *Med12*^{flox} hemizygous males resulted in male embryos containing a *Med12*^{wt} allele with wild-type Med12 expression. All female progeny was heterozygous for the *Med12*^{flox} and *CMV-Cre* alleles leading to excision of the first seven *Med12* exons and generating the *Med12*$^{\Delta 1\text{-}7}$ allele. Because of random X-chromosome inactivation some cells failed to express Med12. Neural tube defects of the female embryos are show and the gray spots represent the mosaic expression of the excised *Med12* allele.

3.3 Results from publication 2

3.3.1 *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre* female embryos have mosaic expression of Med12

In contrast to the general phenomenon which accompanies autosomal heterozygosity, a decrease by approximately 50% of the Med12 protein amount in each cell of *Med12* heterozygous females was not be expected here. Instead, embryos with a mosaic expression of Med12 were anticipated. The reason for this is the process of random X-chromosome inactivation. This embryonic event that occurs in female mammalian embryos solves the issue of double dosage of X-linked genes between female and male cells by inactivating one of the X-chromosomes. Inactivation of the X-chromosome starts at the time of implantation and results in highly heterochromatic condensation incompatible with gene transcription (Clerc and Avner, 2006). Therefore, depending on which chromosome was inactivated, some cells of *Med12* heterozygous females expressed *Med12* from the wild-type copy at standard protein levels, while other cells transcribed the *Med12* gene from the Cre-excised X-chromosome and produced no Med12 protein. In figure 1 of the published article the experiments confirming these predictions are shown.

3.3.2 Mosaic expression of Med12 results in wide phenotypic variation

A wide variety of defects was observed in *Med12* heterozygous females, which confirmed the cellular mosaicism in these embryos. Some displayed severe phenotypes in crucial developmental steps such as cardiac development and axis elongation and were developmentally arrested at E8.5. At E9.5 fully resorbed embryos with only decidual tissue remaining were found, suggesting embryonic death shortly after gastrulation.

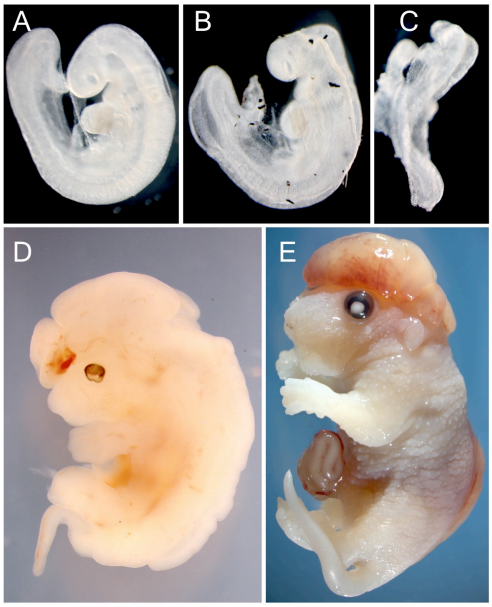

Figure 3.2 Phenotypic variation in *Med12* heterozygous females

A-C embryos at E9.5. (A) Male *Med12*$^{wt/Y}$*;CMV-Cre* embryo with normal embryonic development. B-E *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre* heterozygous females (B) embryo with open neural tube (C) *Med12* heterozygous female with severe embryonic arrest. (D) Fetus at E13.5 with craniorachischisis. (E) Fetus at E16.5 showing exencephaly and spina bifida.

Other embryos developed further and, although they died perinatally, were found alive at E17.5. Phenotypic identification of such mutant fetuses was facilitated by the presentation of severe neural tube closure defects (NTDs) that are known not to interfere with embryonic growth and survival.

The difference between the observed phenotypes in genotypically similar embryos is explained by the difference in contribution of cells expressing the *Med12*$^{\Delta 1\text{-}7}$ allele among the embryos, resulting in normal or reduced Med12 expression. Which cells express one or the other allele and also in which tissues different contributions occur is crucial for the phenotypic outcome. These data can be found in table 1, figure 2 and 3 of the publication and some examples of the phenotypic variety can be seen above in Figure 3.2.

3.3.3 NTDs of *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre* heterozygous females

Both publications described thus far clearly show that Med12 is absolutely necessary for neural tube closure. All *Med12*^{hypo} and *Med12*$^{\Delta 1\text{-}7}$ embryos, despite variable penetrance for other phenotypic abnormalities, had NTDs. NTDs are also very common in humans. The incidence

rate of NTDs across the human population is around 1 in 1000 births, placing neural tube closure at the top of most common birth defects.

Depending on which part of the neural plate remains open, NTDs will have different designations and consequences for the fetus (Figure 2.4). Failure to close caudally to closure point 1 results in spina bifida, which can cause difficulties in locomotion and might affect cognitive capacities but is not necessarily lethal. When closure point 2 fails, the brain tissue is exposed and this leads to death upon birth. In mice this is known as exencephaly, while in humans it is called anenchephaly because of collapse of brain tissue. Finally if point 1 fails to initiate closure then the complete body axis will remain open and cause the lethal disease known as craniorachischisis (CRS). Reflecting their mosaicism, all of these defects could be found in *Med12* heterozygous females. Occurrence ratios for the different phenotypes can be found in table 2 and several examples of defective embryos can be found throughout the publication and in Figure 3.2.

3.4 Discussion of publication 2

The experiments in this publication show that the *Med12*^{flox} allele can be used for *in vivo* Cre-mediated recombination leading to generation of effective *Med12* null embryos where functions of this gene can be studied. The different *Med12* models described thus far: *Med12*^{hypo}, *Med12*$^{\Delta 1\text{-}7}$ and *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre* implicate Med12 as a crucial coregulator in several developmental processes. While its involvement in canonical Wnt and Wnt/PCP signaling is now established and can explain some of the observed developmental abnormalities, others must be explained by its involvement in the disruption of other signaling pathways. To clarify this, similar matings to those described here, i.e. *Med12*^{flox} mice mated to a Cre-expressing strain, which utilizes tissue specific or inducible promoters for expression of the recombinase, will allow for the separation of phenotypes based on tissues, and can bypass the problems of early lethality.

Generation of *Med12* heterozygous females differs slightly from the tetraploid aggregation-derived *Med12*^{hypo} and *Med12*$^{\Delta 1\text{-}7}$ embryos in that natural matings were employed. Due to the many controls performed, the results from Publication 1 remain valid. Nonetheless, it is encouraging and reassuring that similar defects are seen in embryos from both publications. These include: NTDs, axis truncation, and defective heart formation.

This work further extended the gene-specific functions of Med12 by showing its relevance for neural tube closure and for the etiology of NTDs that also affect human embryos. This justifies a screen for mutations on X-chromosome-linked human *MED12* gene in families affected with NTDs in order to investigate whether mutations exist and are associated with a higher disease risk.

Study of *Med12*$^{\Delta 1\text{-}7}$*;CMV-Cre* females will also be useful to clarify the role of Med12 in Wnt/PCP signaling. All mouse models with CRS described so far are mutants for genes that belong to this signaling pathway. Together with the evidence from Publication 1 showing mislocalization of the Prickle1 protein in *Med12*^{hypo} mutants, it is clear that Med12 is essential for establishment of the signaling pathway. Some *Med12* heterozygous females survive up to late

developmental stages and therefore can be used to verify the alignment of the stereocilliary cells of the cochlea. These cells in the vertebrate inner ear are the best-characterized example of Wnt/PCP signaling and if a disruption of the pathway occurs, cells become misaligned during embryogenesis. Similar to what happens in the *Drosophila melanogaster* wing it is known which PCP components should be located at the different poles of the stereocilliary cells (Etheridge et al., 2008; Wang and Nathans, 2007). Studying localization of PCP components in *Med12* heterozygous females will therefore clarify whether the pathway disruption affects localization in a general fashion or if only a few proteins are affected.

4 Unpublished results - Role of Med12 during limb development

Contributors

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4.1 Experimental contributions

Daniela Roth helped perform some of the WISH experiments.

Manuela Sholze prepared some of the probes for WISH.

Heinrich Schrewe supervised and helped planning the experiments.

All other experiments were done by me including planning and supervision of mouse matings, genotyping, phenotypic and histological analysis, skeletal and X-gal stainings, and some of the whole mount *in situ* hybridizations.

4.2 Introduction to limb development

The vertebrate limb is an excellent model to study formation of body plans, growth, patterning, and how precise arrangements of specialized cells and tissues arise. The limb bud consists of an ectodermal epithelial layer enclosing undifferentiated mesenchymal cells, and originates from the lateral plate mesoderm. In mice, limb bud formation occurs at E9.5. As limb outgrowth proceeds, mesenchymal cells start differentiating into tissues such as cartilage and bone. An intricate and tightly regulated gene network is necessary to ensure correct formation of all structures that compose the mammalian limb. The digits and the limb skeleton are among such structures and many genes from different signaling pathways are known to regulate these processes (Duboc and Logan, 2009). From the experiments described in the previous chapters it is clear that Med12 acts as an *in vivo* coregulator for transcription factors of several murine embryonic signaling pathways and, therefore, it is highly plausible that it is also necessary for limb development. Using the limb as a model, other regulatory functions of Med12 can be elucidated which may lead to identification of novel transcription factors that use Med12 to regulate gene transcription.

4.2.1 Signaling centers and molecules controlling limb patterning and growth

Limb growth is coordinated upon three different axes: proximal-distal (PD) running from the shoulder to the digits; anterior-posterior (AP), from thumb to little finger and dorsal-ventral (DV) from the back of the hand to the palm. Correct patterning of this structure is established by molecules specifically expressed in each of these axes, which contain specialized signaling centers like the apical ectodermal ridge (AER) for the PD axis and the zone of polarizing activity (ZPA) for AP specification (Figure 4.1). It is not yet fully clear how the different signaling pathways precisely interact to coordinate patterning and growth of the vertebrate limb, but years

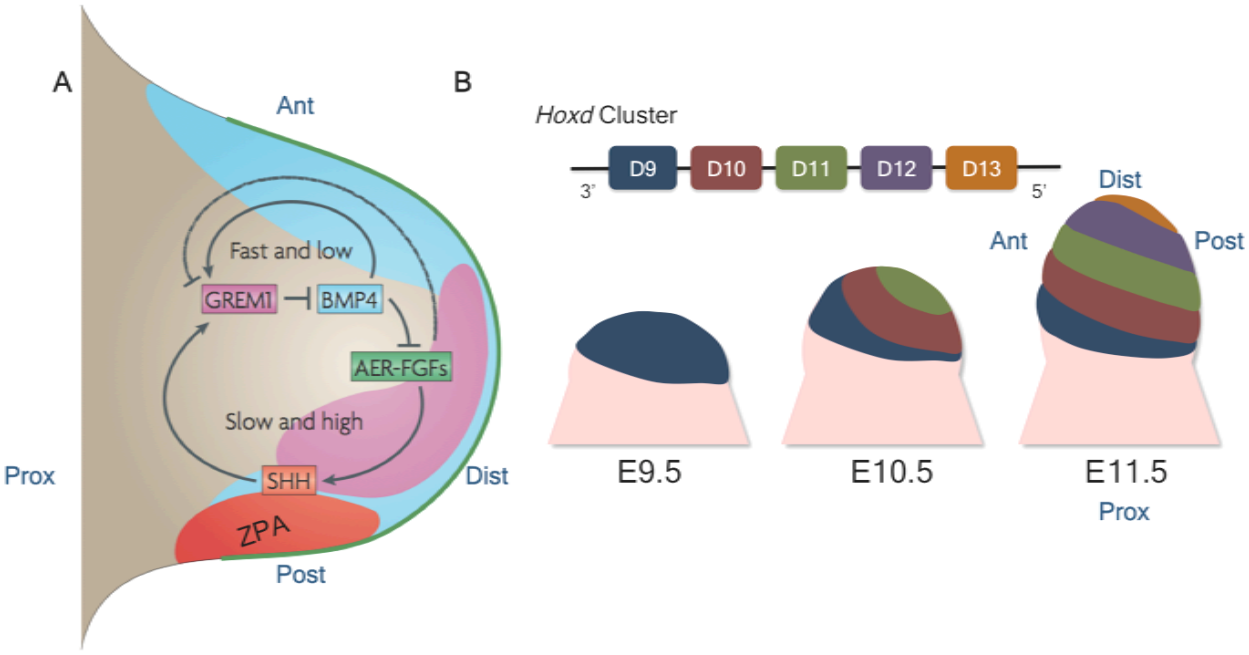

Figure 4.1 Regulation of limb patterning and growth

(A) Molecules involved in AP and PD patterning of the vertebrate limb. Fgf molecules from the apical ectodermal ridge (AER) control expression of Shh (orange) from the zone of polarizing activity (ZPA). Shh in turn maintains, through a feedback mechanism, expression of AER Fgfs (green). The feedback mechanism in the mesenchymal cells of the limb is controlled by the levels of Bmp4 (blue) and its agonist Grem1 (purple). Figure modified from (Zeller et al., 2009). (B) Genes of the *Hoxd* cluster are key regulators of limb development. Genes located most upstream in the cluster are expressed first and at E11.5 occupy proximal anterior regions while downstream genes are activated later and mark posterior distal areas of the limb.

of genetic and tissue-fate experiments have identified several important players in these processes as well as their function (Duboc and Logan, 2009).

The AER, an epithelial tissue resulting from thickening of the ectoderm, is located at the distal most tip of the limb and separates the ventral from the dorsal side. Several ligands of the fibroblast growth factor (Fgf) family are expressed at the AER and are responsible for inducing proliferation of the undifferentiated limb mesenchymal cells. This regulates expansion of the limb along its PD axis and accordingly, mutations in AER *Fgfs* cause limb truncations (Mariani et al., 2008).

The ZPA is located at the distal, posterior margin of the limb, and is a source of the secreted molecule sonic hedgehog (Shh), which is a key determinant for AP patterning. Fgf signals from the AER are required for maintenance of Shh expression, which in turn, through a

positive feedback mechanism, contributes to a continuously active Fgf status in the AER (Niswander et al., 1994). Components of the feedback mechanism include members of the bone morphogenetic protein family (Bmps), such as Bmp4 and its agonist gremlin 1 (Grem1) (Zeller et al., 2009). An overview of these interactions can be seen in Figure 4.1. Also contributing to AP patterning and to ZPA activity are genes of the *HoxD* cluster. These genes, which have spatially and temporally collinear expression (Figure 4.1), are essential for activation of *Shh* in the ZPA but at later stages become dependent on Shh for their expression in the posterior developing limb. The most 5' gene of the *Hoxd* cluster, *Hoxd13* is expressed at the posterior most distal tip, while more 3' genes (i.e. *Hoxd10*) mark anterior proximal regions (Tickle, 2006).

Finally, the third signaling center in the developing vertebrate limb is the non-AER dorsal ectoderm. This tissue is responsible for DV patterning via Wnt signaling. Wnt7a is produced by the dorsal ectoderm and induces expression of the LIM-homeobox gene *Lmx1b*. Dorsal ectoderm signaling can also impinge on the ZPA and Shh expression (Hill et al., 2006).

4.2.2 Regulation of skeletogenesis

Formation of cartilage and bone is essential for limb development to proceed. The mouse skeleton starts developing around E12.5 when the first mesenchymal cells begin to condense. A few bones, such as the flat bones of the skull and the clavicle are formed through the process of intra-membranous ossification. The vast majority of bones are formed through the alternative mechanism, called endochondral ossification where a cartilaginous anlage precedes the formation of calcified bone. At the initiation of endochondral ossification, mesenchymal cells aggregate, condense and start to differentiate into chondrocytes (cartilage producing cells). Cells in the periphery of the condensation form the perichordium. A portion of this tissue can later differentiate into osteoblast (bone producing cell) precursors and form the periosteum. Meanwhile, chondrocytes in the center of the condensation exit the cell cycle, stop cell proliferation and become hypertrophic. Upon maturation, such cells produce a mineralized

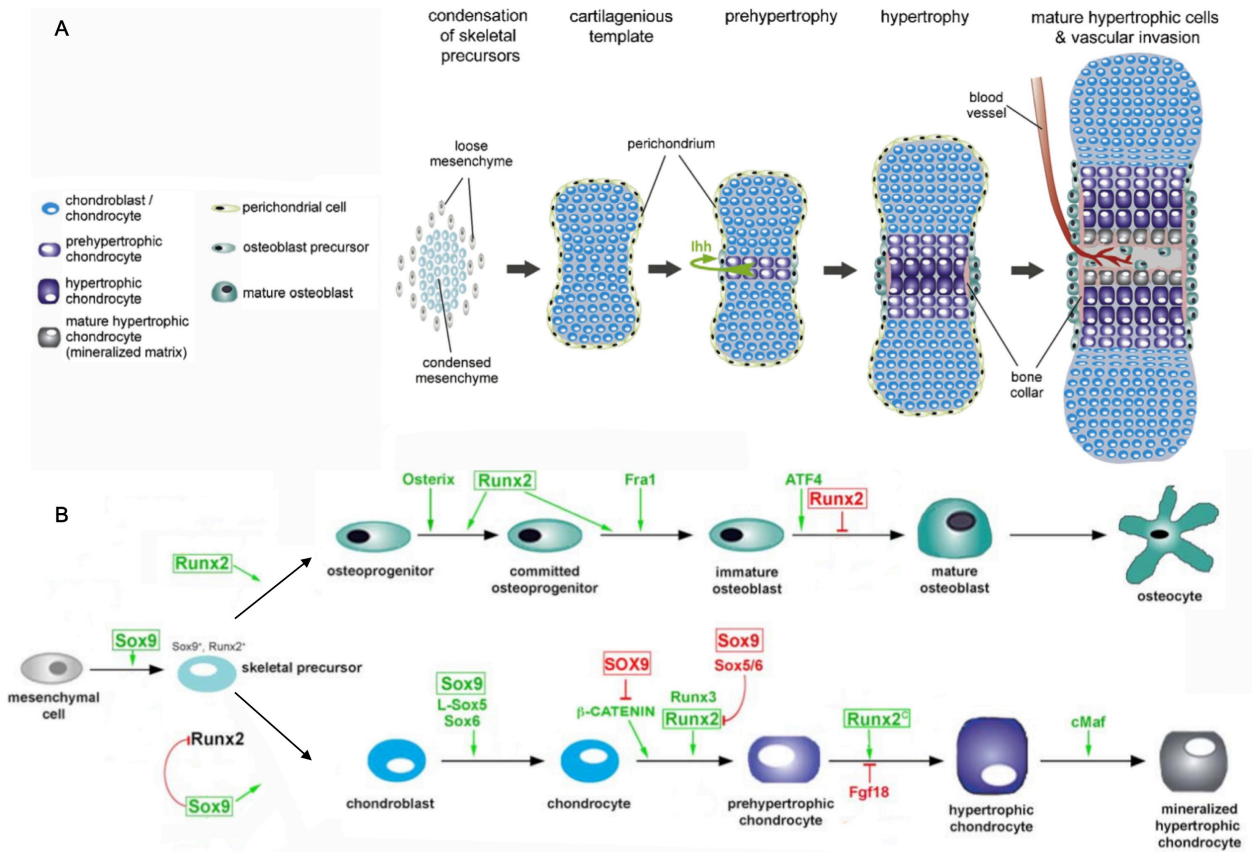

Figure 4.2 Transcriptional network controlling endochondral ossification

(A) The process of endochondral ossification that occurs in most bone starts with condensation of mesenchymal cells, that later become cartilaginous. These cells become hypertrophic, mineralize, and induce formation of osteoblasts through secretion of Indian hedgehog (Ihh). Furter information can be found in the text. (B) Transcription factors controlling the stages of cartilage formation (bottom) and ossification (top). Key molecules that contribute to a certain differentiation step are represented in green and transcription factors that have a negative influence in the process are shown in red. Figure modified from (Hartmann, 2009).

extracellular matrix that, after cell-death by apoptosis, is used as a template for trabecular bone formation by osteoblasts. The trabeculae are highly vascular tissues containing red bone marrow and are found at the end of long bones such as those of the limb. Besides providing a physical scaffold for bone formation, chondrocytes in the pre-hypertrophic stage produce signaling molecules like Indian hedgehog (Ihh), which induce osteoblast differentiation of cells in the surrounding perichondium (Hartmann, 2009). The process of endochondral ossification is illustrated in Figure 4.2.

Several transcription factors have already been identified that coordinate the different steps of endochondral ossification. The earliest cell marker for a chondrogenic fate is the TF Sox9, which has been shown to drive expression of extracellular matrix proteins such as

collagen (coded by the *Col2a1* gene) and matrilin. Both are required for the formation of cartilaginous structures. Expression of these proteins is accomplished in connection with two other members of the Sox family of transcription factors, Sox5 and Sox6, whose transcription is also controlled by Sox9. The switch to a hypertrophic status is then controlled by Runx2 and β-catenin. Premature induction of hypertrophy is likewise controlled by Sox9, which can keep cells in the proliferative chondrocyte stage. cMaf has been, so far, the only protein shown to be essential for the terminal differentiation of chondrocytes (Karsenty, 2008).

As mentioned above, formation of osteoblasts in the perichondrium is regulated by signals from the chondrocytes. Ihh is one of these signals, and induces expression of *Runx2*, which then initiates the specification of the osteogenic line. Upon *Osterix* expression, cells become fully committed to differentiate into osteoprogenitors. Maturation of osteoblasts is then achieved following inhibition of *Runx2* and activation of genes such as *Fra1* and *Atf4*.

4.3 Materials and Methods used for these experiments

Most of the techniques and reagents used in the experiments reported in this chapter have already been described in the Materials and Methods sections of the two published articles described in chapters 2 and 3. Methodology specifically used in this chapter will be described bellow.

4.3.1 Mouse breeding and genotyping

The *Med12*^{flox} line and primers for the genotyping were already described in Publication 1 and 2. The *Prx1-Cre* line was generated in the laboratory of Cliff Tabin (Logan et al., 2002) and the following specific primers for this transgene were designed: 5'-GTTGGCAAAGGGGTTTTCTT and 5'-ACGGACAGAAGCATTTTCCA. The *ROSA26R* reporter line was described in (Soriano, 1999) and the same genotyping primers were used in this study to verify for homozygosity of the *ROSA26R* line. Homozygous *Med12*^{flox} females were mated with homozygous *ROSA26R* males and the double heterozygous progeny were intercrossed to originate double homozygous female mice, which were then mated with *Prx1-Cre* males. These embryos were used for X-gal staining. All other experiments were performed with progeny of *Med12*^{flox} matings with *Prx1-Cre*.

4.3.2 X-gal staining

Embryos were dissected in PBS and fixed in 4%PFA/PBS at 4 ^{o}C for one hour. Specimens were then rinsed three times at RT in rinse buffer (5 mM EGTA, 0.01% deoxycholate, 0.02% NP40, 2mM $MgCl_2$, in PBS). Meanwhile, the staining buffer was prepared containing 5 mM potassium ferricyanide (Merck) and 5 mM potassium ferrocyanide (Merck), in rinse buffer. To this X-gal (40 mg/ml in dimethylformamide) was then added to a final concentration of 1 mg/ml, and

the prepared staining buffer was then filtered. Staining was done o/n at 37 °C in the dark. After staining, embryos were washed 3 times with PBS and refixed in 4%PFA/PBS.

4.3.3 Probes used for WISH

The protocol for whole mount *in situ* hybridization followed here was identical to what was used for Publication 1. Probes for the following genes were obtained from the MAMEP database (http://mamep.molgen.mpg.de/): *Hoxd10, Hoxd12, Lmx1b, Fgf8, Shh, Col2a1* and *Myod1*. Probes for *Bmp4* and *Sox9* were a kind gift from Ulrique Dohrmann (Max-Planck Institute for Molecular Genetics, Berlin) and *Grem1* from Sigmar Stricker (Max-Planck Institute for Molecular Genetics, Berlin)

4.3.4 Limb micromass cultures

A protocol adapted from (Karamboulas et al., 2010) was followed. Briefly, embryos were isolated in PBS at E11.5. The distal portion of the limbs was amputated from the embryo and collected in HBSS (Thermo Scientific). A digestion followed with Dispase (Roche) at a final concentration of 3 mg/ml at 37 °C for 15 min. This digestion was required for removal of the ectodermal tissue. Limbs were then washed in HBSS and an additional digestion was done for 45 min at 37 °C in a solution containing 0.1% Collagenase Type IA (Roche), 0.1% Trypsin and 5% FCS in PBS. Single cell suspension was then obtained through vigorous pipetting and by filtration through a 30 μm cell strainer. Cells were then counted with a hemocytomer and resuspended in culture medium (10% FCS, 2 mM Glutamine, in DMEM:F12 medium [Thermo Scientific]) to allow seeding of 10 μl drops containing $1.5x10^5$ cells. After two hours, fresh medium was applied to the cells, which were then cultured at 37 °C 7.5% CO_2 for 6 days. Medium was changed every 48 hours. Micromass cultures were washed twice and fixed in Kahle's Fixative (absolute alcohol, 40% formalin and glacial acetic acid in the ratio of 15:6:1). Three washes with PBS were performed and specimens were stained o/n with 1% alcian blue,

pH 1.0. To remove excess staining solution washes with water followed, and micromass cultures were photographed using a binocular microscope with attached camera.

4.3.5 *In vitro* culture of limb explants

Forelimb and hindlimb buds from E11.5-12.5 embryos were amputated using sharp forceps in ice-cold PBS/5% FCS and placed in holding medium (L15 Leibowitz medium [Gibco]/1x MITO+ serum extender [BD Biosciences]/1x pen/strep). Limb buds were allowed to sit in holding medium for 30 min to 2 hours. Limbs were then placed on PET track-etched cell culture membrane inserts (8 µm pore size, Falcon) with the palm face-up. The inserts were transferred into a 6-well tissue culture plate containing explant medium (DMEM/F12 (1:1)/5x MITO+ serum extender/1x pen/strep/10% FCS). Explants were cultured on the membranes at the air-liquid interface in an incubator at 37 °C under an atmosphere of 7.5% CO_2 for 4 days. Explant medium was replaced every 48 hours. After 4 days in culture, explants were stained with alcian blue similarly to micromass cultures.

4.4 Genetic models of *Med12* used in these experiments

The limb is an excellent model system for study of differentiation and embryonic patterning processes. The experiments with *Med12*^{flox} and *CMV-Cre* mice (Chapter 3) have shown that the floxed allele can be excised *in vivo* allowing the study Med12 functions. To investigate the role of Med12 during limb formation, *Med12*^{flox} mice were mated with mice carrying a *Prx1-Cre* transgene. This transgene contains a fragment of the distal promoter of *Prx1* (Figure 4.3A) and leads to expression of Cre recombinase in the mesenchymal cells of the limbs immediately at E9.5, when limb buds are first formed (Logan et al., 2002).

In the experiments reported in this chapter, *Med12*^{flox} females were mated with

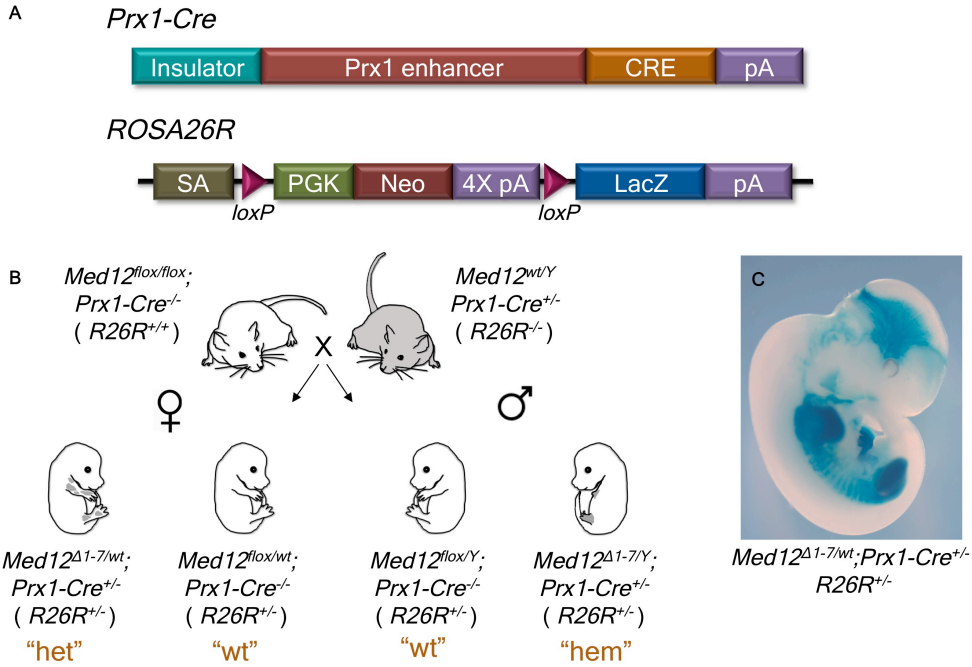

Figure 4.3 Mouse lines and matings used to study the function of Med12 in the developing mouse limb.

(A) The *Prx1-Cre* transgene used in these experiments contains the following elements: an insulator that protects transcription of Cre by blocking harmful interferences from the integration site of the transgene; a portion of the *Prx1* distal promoter that leads to expression in the limbs; the *Cre* coding sequence and a polyA signal. The *ROSA26R* allele is also represented. An expression cassette for *LacZ* was introduced in the *Rosa26* locus together with a floxed-neomycin expression cassette containing a quadruple polyA sequence. Upon Cre-mediated recombination the cassette is excised and allows transcription of *LacZ*. (B) Breeding scheme used for the production of male mice without Med12 in the limb buds (hem), with wild-type expression of the protein (wt) and heterozygous Med12 mosaic females (het). (C) Whole mount X-gal staining of a E12.5 *Med12*$^{\Delta 1\text{-}7/wt}$*;Prx1-Cre*$^{+/-}$*;R26R*$^{+/-}$ heterozygous female showing tissues with the excised *Med12* allele.

heterozygous male *Prx1-Cre* mice. Some of the embryos generated by this mating carried no copy of the *Prx1-Cre* transgene and had, therefore, unexcised *Med12*^{flox} alleles. Such mice were named wild type (wt), as they were capable of normal Med12 expression. Additionally, litters from these matings contained embryos carrying one copy of the *Prx1-Cre* allele, which led to *Med12* excision in cells of the limb mesenchyme. All male embryos generated were hemizygous (hem) for the floxed allele and therefore Cre-mediated excision led to limb buds where the mesenchymal cells were unable to express Med12. Female embryos, on the other hand, had a floxed allele and a wild-type X-chromosome. Similarly to what was described in the previous chapter, due to random X-chromosome inactivation, limbs of such heterozygous females expressed Med12 in a mosaic fashion (Figure 4.3B).

To verify the expression domains of the *Prx1-Cre* transgene, *Med12*^{flox} mice were mated with the *ROSA26-LacZ* reporter line (R26R). This line was generated by inserting a *LacZ* conditional expression cassette into the *ROSA26* locus. Upon Cre excision, the *LacZ* gene is expressed producing the β-galactosidade enzyme, which in the presence of a substrate such as X-gal, allows visualization of the cells where excision occurred (Soriano, 1999). Mice homozygous for the *ROSA26R* and *Med12*^{flox} alleles were generated and then mated with heterozygous *Prx1-Cre* males. The progeny of these embryos contained two floxed alleles, *Prx1-Cre* and *Rosa26R*. X-gal staining allowed visualization of the cells that were able to express β-galactosidase and this indicated which cells possessed the excised *Med12*$^{\Delta 1\text{-}7}$ allele. This experiment helped to confirm the expression of the *Prx1-Cre* transgene. As it has been described, excision was detected not only in the mesenchyme of the limb buds, but also in the head mesenchyme and in the forming ribs (Logan et al., 2002). This experiment shows that *Prx1-Cre* mice leads to excision of the *Med12*^{flox} allele in a tissue specific manner, which will help to identify potential function of the Med12 during limb development.

4.5 Results of the study

4.5.1 Med12 is necessary for limb formation

Matings of *Med12*^{flox} female with *Prx1-Cre* males clearly showed that Med12 is crucial for limb development. Fetuses carrying the *Prx1-Cre* transgene and the floxed *Med12* allele were found alive at E17.5 and displayed a clear phenotype in limb formation. As seen in Figure 4.4A, both forelimbs and hindlimbs of hemizygous males were extremely truncated and only a very rudimentary structure could be seen. Hindlimbs of heterozygous females had a regular size and extension although formation of digits was abnormal when compared to wild-type controls. Forelimbs from the female fetuses were relatively smaller than those of wild-type littermates, had a bent shape and extremely affected digits. Only hemizygous males were chosen to proceed

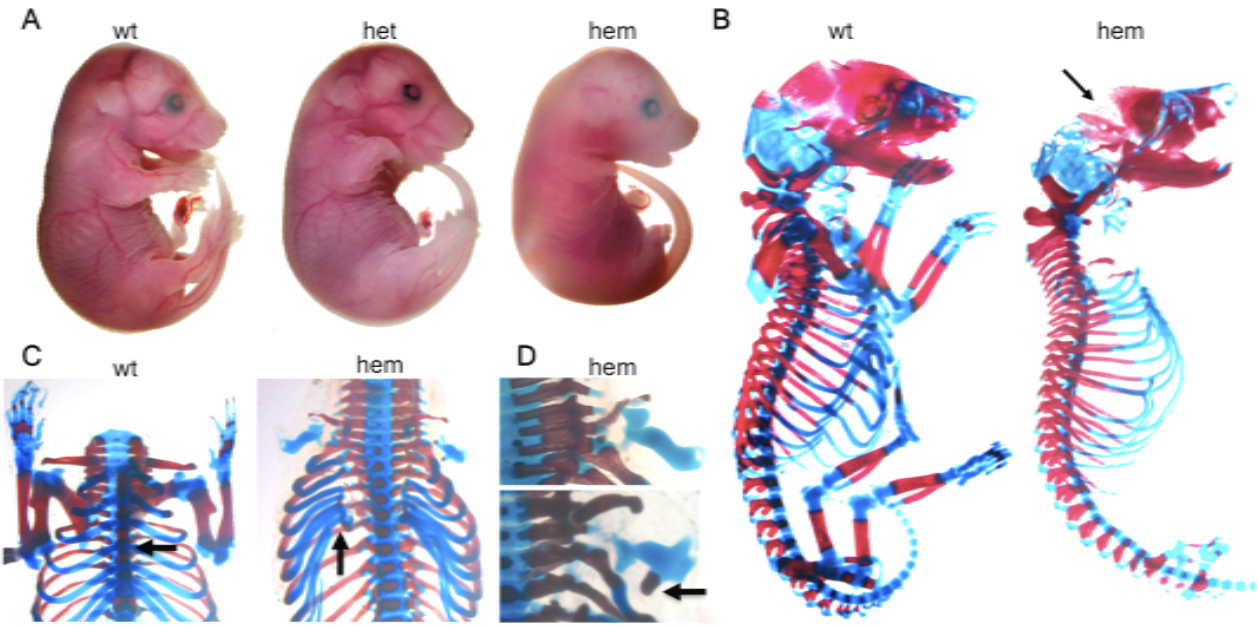

Figure 4.4 Med12 is essential for formation of the limbs

(A) Gross morphology of wild type, heterozygous and hemizygous fetuses at E17.5. (B) Alcian blue and alizarin staining of wild-type and hemizygous *Med12* embryos at E17.5. Arrow points to skull defects seen in the absence of Med12 (C) The rib cage of *Med12* mutants does not close at the midline and lacks the sternum (arrows). (D) (top) Dorsal view showing the rudimentary scapula and humerus of hemizygous mutants. (bottom) Ventral view of a mutant with a small ossification in the humerus.

with the analysis of the limb phenotype observed in the absence of Med12 because of the smaller phenotypic variability among specimens. Heterozygous females expressed Med12 in a mosaic fashion and the observed differences in severity of defects could complicate the studies. Fetuses were then stained with alcian blue (for cartilage) and alizarin red (for bone), which demonstrated that the rudimentary forelimbs of hemizygous males contained virtually no skeletal structures. The hindlimbs, on the other hand, had ossified bones in the pelvic region and a cartilage femur-like bone Figure 4.4B. Closer analysis of the embryos revealed that the ribs of heterozygous mice did not fuse at the midline and the sternum was not formed (Figure 4.4C). This is not compatible with *ex utero* survival, as hemizygous male neonates would not be able to breathe. In Figure 4.4D it can also be seen that the forelimbs of hemizygous mice could form a scapula (although extremely reduced in size) and a rudimentary cartilaginous humerus that, in some embryos, developed a small ossified structure. Additionally, *Med12* hemizygous embryos presented with a clear deficiency in formation of the parietal bone, in line with the observation that the floxed allele was also excised in the cell population that gives rise to this structure (Figure 4.4B).

4.5.2 Normal expression of patterning genes and growth signaling molecules

A time course analysis revealed that the morphological differences between Med12 hemizygous mutants and wild-type controls were first visible at E12.5. At this time point mutant limbs were already relatively smaller than controls and digit formation had not started. As discussed in the introduction to this chapter, several signaling pathways control the outgrowth of the vertebrate limb. WISH was used to verify if the signaling centers responsible for limb patterning expressed the appropriate molecules at E11.5, the time point where the Med12 phenotype seems to be established (Figure 4.5).

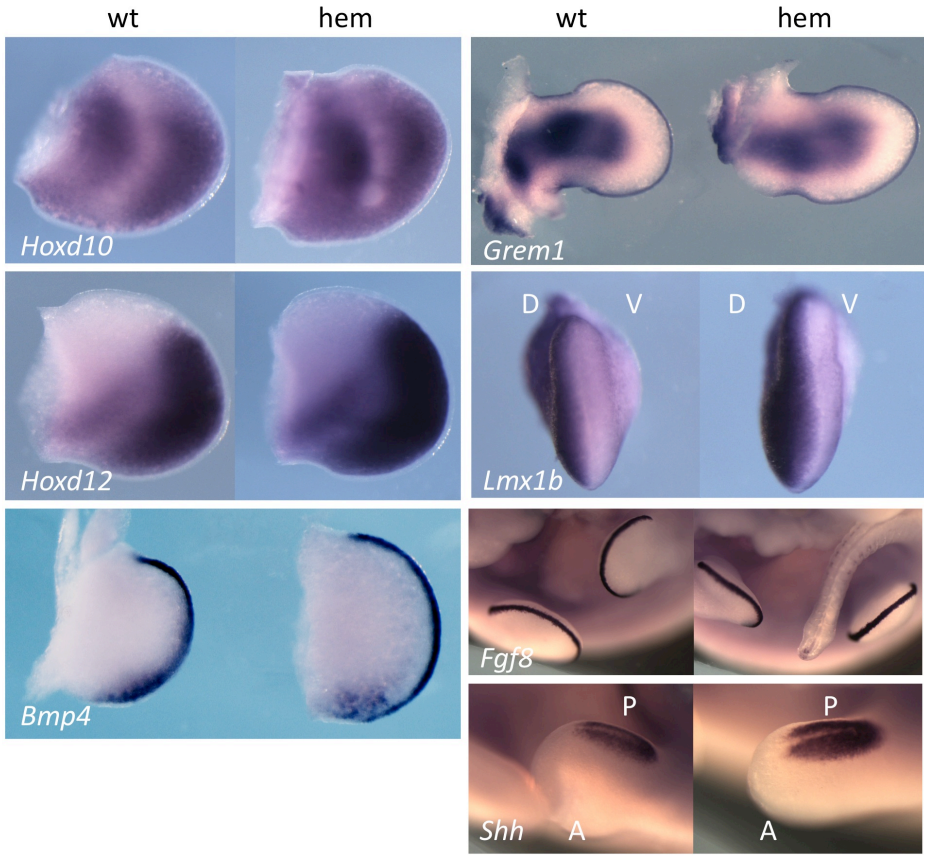

Figure 4.5 WISH to genes involved in limb patterning

Med12$^{\Delta 1\text{-}7/Y}$*;Prx1-Cre* hemizygous embryos and wild-type controls at E11.5 were used in WISH to investigate expression of genes involved in limb patterning and growth.

Fgf8 is the main Fgf expressed by the AER and is sufficient for induction of this signaling center and limb outgrowth. Its expression was found to be unaltered in the absence of Med12. Similar results were obtained from analyzing expression of *Shh* from the ZPA. This signaling center was also correctly established in the posterior margin of mutant embryos at 11.5. Similarly, *Grem1* and *Bmp4*, which are crucial for maintaining crosstalk between the AER and ZPA also showed an unaltered expression pattern at this stage. Additionally, dorsal-ventral patterning was correctly established in Med12 mutant limbs judging by expression of the dorsal marker *Lmx1b*. Expression of two members of the *HoxD* cluster, *Hoxd10* and *Hoxd12* was found to be undisturbed upon Cre-mediated excision of *Med12* in the developing limbs.

These results indicate that disturbed limb patterning or abnormal functioning of the limb signaling centers are not the cause behind the limb phenotype observed in *Med12*$^{\Delta 1\text{-}7/Y}$*;Prx1-Cre* embryos.

4.5.3 Med12 is essential for chondrogenesis

Since limbs of *Med12*$^{\Delta 1\text{-}7/Y}$;*Prx1-Cre* hemizygous mice establish all signaling centers required for limb growth and express correctly the major patterning molecules, a micromass culture experiment was designed to investigate if abnormal chondrogenesis led to the limb defects. These experiments are performed by isolating the undifferentiated mesenchymal cells of the limb buds from E10.5-E12.5 embryos. Cells are then cultured in high-density conditions and this induces the formation of chondrocytes, which can become hypertrophic and mineralize. Alcian blue staining allows the quantification of the ability to form cartilage. Mesenchymal cells from E11.5 mutant and wild-type embryos were isolated and cultured. Although cells of hemizygous mutants were able to form condensations, their chondrogenic potency was lost and even under conditions of highly dense cell-cell interactions they failed to initiate mineralization. Limb bud cells of heterozygous female embryos developed a few chondrogenic condensations but in extremely reduced fashion in comparison to wild-type embryos (Figure 4.6A).

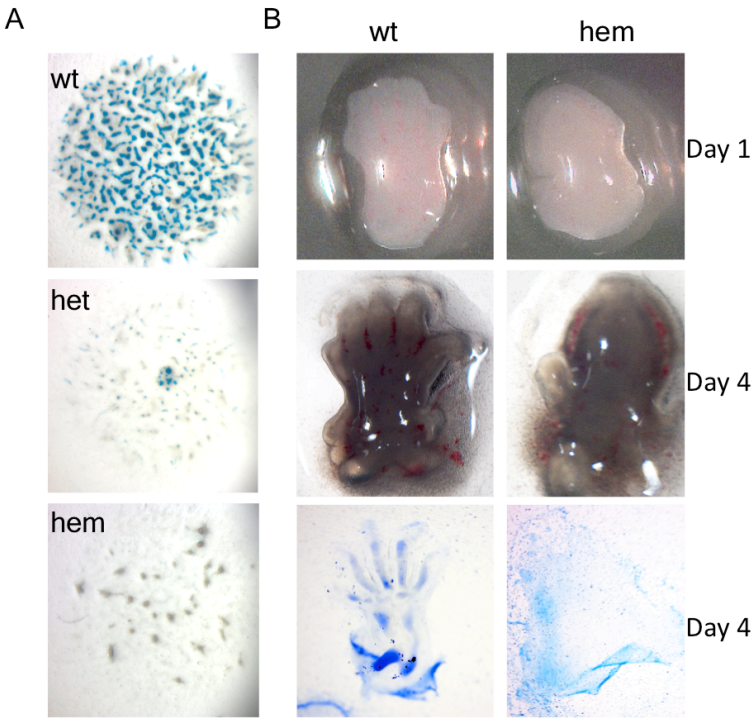

Figure 4.6 Med12 is essential for cartilage formation

(A) Micromass cultures of hemizygous and heterozygous *Med12* mutants and wild-type controls. Cells of heterozygous embryos show condensations but have a severely compromised capacity to initiate chondrogenesis in comparison to cells of wild type controls. In the absence of Med12 no cartilage sites were found. (B) Limbs of E12.5 embryos were isolated and grown in culture. After 4 days of culture, limbs of *Med12* hemizygous mutants failed chondrogenesis.

Limb explant cultures also demonstrated that Med12 is essential for initiation of endochondral ossification. This assay, where the distal portion of E11.5 or E12.5 limbs is cultured *in vitro*, allows careful monitoring of limb formation and chondrogenesis. Limbs isolated either at E11.5 or E12.5 from Med12 mutants failed to develop cartilage even after four days in culture while wild-type controls developed, *in vitro*, a structure that closely resembled the hand skeleton (Figure 4.6B). These results suggest that a block in cartilage formation, an essential initial step in the process of endochondral ossification, causes the limb defects observed in the absence of Med12.

4.5.4 Med12 is an *in vivo* coactivator of Sox9

The experiments described above implicate Med12 as a coregulator involved in the first steps of chondrogenesis. Sox9 is the earliest cell marker for the chondrogenic fate and is responsible for activating expression of genes such as *Col2a1* that are important components of

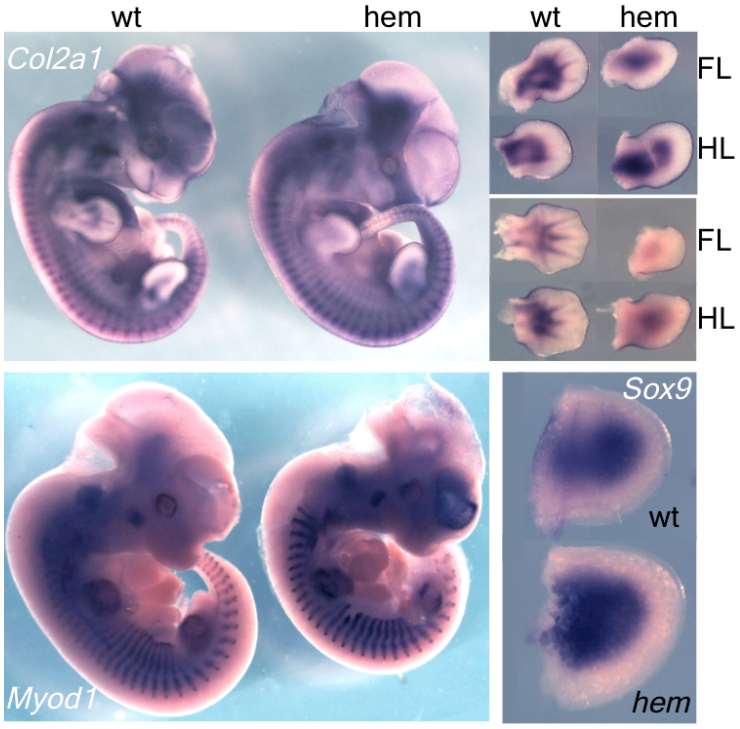

Figure 4.7 Med12 is required for correct expression of the Sox9 target *Col2a1*.

WISH with a *Col2a1* probe at E11.5 (top). Insets highlight the expression of the gene in forelimbs (FL) and hindlimbs (HL) both at E11.5 and E12.5. The *Myod1* gene, which is responsible for inducing myogenic lineage, is correctly expressed in the absence of Med12 at E11.5 (bottom). *Sox9* expression in the limbs was found at similar levels in Med12 mutants and wild-type littermates.

the extracellular matrix secreted by chondrocytes. The human SOX9 and MED12 can physically interact and, in zebrafish, these proteins were shown to act together for activation of genes involved in chondrogenesis. A clearly deficient *Col2a1* expression in limbs that lacked Med12 is in line with a role for Med12 as a coactivator for Sox9 during transcriptional activation of chondrocyte specific genes. At E11.5, while hindlimbs of mutants and wild-type controls showed no difference in the expression domain of *Col2a1,* forelimbs of *Med12* hemizygous mutants had an already compromised and delayed expression. Wild-type controls expressed *Col2a1* in the future sites of digits formation but mutant embryos failed to do so (Figure 4.7A). The more severe *Col2a1* misexpression in the forelimbs might explain why at later stages these structures have a much more deficient skeleton. At E12.5 the abnormal *Col2a1* expression in Med12 hemizygous could be easily seen in all limbs.

To verify that only targets of Sox9 are affected and that this TF is still expressed in the developing limbs of Med12 mutants, a *Sox9* WISH was performed. *Sox9* expression at E11.5 in controls was undistinguishable from Med12-deficient embryos both in forelimbs and hindlimbs of E11.5 embryos (Figure 4.7C), demonstrating that the chondrogenic pathway is very likely disrupted at the activation step of Sox9 target genes. Additionally, illustrating the fact that *Med12*$^{\Delta 1\text{-}7/Y}$*;Prx1-Cre* embryos did not have a generalized defect in all cells of the developing limb, *Myod1* expression was also assayed by WISH and showed that myogenesis (formation of muscular tissue) was not perturbed (Figure 4.7B).

4.6 Discussion of the study

The experiments described in this Chapter demonstrated that the *Med12*^{flox} line can indeed be used for tissue-specific deletion of *Med12*. Using the *Prx1-Cre* transgenic mouse line it was possible to generate a loss-of function mutation affecting only mesenchymal cells of the limb bud, the rib cage, and the head mesenchyme thus allowing bypass of the early embryonic lethality seen in *Med12* mutants described in the previous chapters. This system established Med12 as a crucial coregulator for limb development and chondrogenesis.

Correct limb development stalled at E11.5 in the absence of Med12. Up to this stage, growth and patterning are the main events that occur in the vertebrate limbs. *Med12* mutant limb buds showed no defect in size, and expression of marker genes known to coordinate the establishment of patterning signaling centers was not affected. The first signs of mesenchymal cells differentiation can be seen at E12.5, when endochondral ossification begins and the first chondrogenic sites appear. This marks the beginning of limb skeletal formation, which is essential for limb development to proceed due to its fundamental structural role. At E12.5, digits can already be recognized. Med12-deficient embryos at E12.5 showed abnormalities in both of these processes. Chondrogenesis was clearly delayed in the absence of Med12, and expression of *Col2a1*, one of earliest cell markers of the chondrogenic lineage was severely compromised. Failure to build the skeleton led to an arrest in limb development, which was especially marked in the forelimbs.

Previous studies have shown that the human MED12 interacts with SOX9 and in the fish *Danio rerio* it is required for activation of Sox9 target genes such as *Col2a1.* These results, together with the observations reported in this Chapter, indicate that Med12 acts *in vivo* as a coactivator for Sox9 and that it is essential for activation of its target genes and the associated processes such as induction of the chondrogenic lineage. *Med12* mutation caused the absence of skeletal structures in the limbs of *Med12*$^{\Delta 1\text{-}7/Y}$*;Prx1-Cre* embryos and limb developmental arrest.

The role of Med12 in induction of the chondrogenic lineage was not limited to the developing limbs. In line to what has been described for the *Prx1-Cre* mouse line, excision of the floxed *Med12* allele occurred also in cells that contribute to formation of the rib cage and the head mesenchyme. Both these cell populations form at later stages skeletal structures, which were affected in *Med12* hemizygous embryos. In the absence of Med12, the sternum was absent thus inhibiting fusion of the developing ribs at the midline and parietal bones of the skull were smaller indicating that the head mesenchyme also performed skeletogenesis deficiently.

It was expected that limb patterning would require Med12. First, limb patterning is a complicated process with an intricate gene regulatory network coordinated by several signaling networks and with complex crosstalk among its components. Second, the experiments reported in the previous chapters established Med12 as a coactivator for one of such regulatory pathways i.e., canonical Wnt signaling and suggested that other important regulators of mouse development use it as an anchor to recruit the Mediator to the promoters of its target genes. Nonetheless, it seems that although Med12 might play a role during patterning of the vertebrate limb it may also be a redundant coactivator for this process. Also intriguing is the differing severity of the Med12 phenotype observed between forelimbs and hindlimbs. Whereas practically no skeletal structures were seen in the former, the latter possessed well-formed, pelvic bones and a relatively developed femur. The cause of this observation remains unknown but it could be related to different kinetics of *Med12* excision between hindlimbs and forelimbs. This theory is supported by the different induction points for their formation in the developing mouse embryo.

In summary, these experiments highlight the benefits of using mouse lines with tissue-specific Cre expression to study embryonic and even adult processes controlled by Med12. They show that Med12 plays a crucial role inducing chondrogenesis by acting as a coactivator for Sox9. The system described here together with *in vitro* methods like micromass cultures provide a useful model to expand on the molecular studies of how the Mediator controls transcription of

target genes activated by transcription factors that target individual subunits of the complex. Cre lines such as the *Col2a1-Cre*, which leads to excision of floxed alleles in chondrocytes (Ovchinnikov et al., 2000) could further elucidate the function of Med12 during skeletal development and identify additional steps regulated by Med12.

5 Final Discussion

5.1 Main conclusions from this thesis

Due to its recent discovery, the importance and function of the Mediator during eukaryotic transcription was largely unknown at the beginning of the decade. Since then, several laboratories from different disciplinary fields have contributed to the current definition of the Mediator as a regulatory hub able to sense the stimuli to which a cell is exposed and convey this information to an appropriate response at the level of transcription. Several subunits of the complex have been shown to work as sensors by specifically interacting with transcription factors working as end points of signaling pathways. Mechanisms and subunits responsible for translating these signals into modifications of the chromatin or direct activation of Pol II machinery have also been described. Nevertheless this is not true for all components of the Mediator and for several subunits, functions remain either unknown or contradictory reports produce confusing arguments. This is the case for Med12, a member of the Cdk8 module, whose task within the complex is itself still not fully understood. Med12 has been linked both to gene-specific activities by serving as an anchor for different transcription factors and to a more general role in the regulation of transcription by the Mediator (Malik and Roeder, 2010; Taatjes, 2010). The experiments described in this thesis aimed at clarifying the *in vivo* function of Med12 by generating and analyzing mouse embryos carrying different mutant alleles (with varying protein expression levels).

Subunits of the Mediator that play a general structural role in the complex are usually required for transcription of almost all genes and are therefore necessary for cell survival. In contrast, proteins of the complex that are used in a more gene-specific fashion, when mutated, lead to less severe phenotypes since fewer cellular processes are affected (Blazek et al., 2005). The developmental defects observed in the mutants described in this thesis show that during mouse development Med12 is not essential for cell viability but plays major regulatory roles in

the establishment of several embryonic structures and in the regulation of specific developmental processes.

Chronologically, a hypomorphic *Med12* allele (*Med12*^{hypo}) was the first to be generated as a by-product of the gene-targeting event in ES cells. Embryos containing this allele had a highly compromised expression of Med12 and survived up to midgestation. ES cells and mice carrying the *Med12*^{flox} allele, which was the end product of the gene-targeting, expressed Med12 at wild type levels, and were instrumental in obtaining a full null allele unable of Med12 protein expression. This was accomplished by Cre-mediated recombination, which promoted excision within the *Med12*^{flox} allele where *Med12* critical exons were flanked by *loxP* sites. The resulting *Med12*$^{\Delta 1\text{-}7}$ ES cells were used to generate embryos lacking Med12 that died at early gastrulation. Matings of the *Med12*^{flox} mouse line with two Cre-deleter lines; *CMV-Cre* and *Prx1-Cre* provided heterozygous embryos with mosaic expression of Med12 and embryos with deficient protein expression at the developing limbs, respectively. Embryos from these two matings were able to develop further and permitted identification of additional embryonic processes controlled by Med12.

Experiments with embryos carrying the different alleles established Med12 as an essential transcriptional coregulator during mouse development. Specifically, Med12 null embryos highlighted the critical role of this protein during formation of mesoderm and induction of EMT. The hypomorphic embryos with a severely compromised, but still detectable Med12 expression, developed two additional days in comparison to null embryos and demonstrated the importance of Med12 in formation of mesoderm and elongation along the embryonic posterior axis. Additionally, hypomorphic mutants showed that Med12 is necessary for establishing the somitic clock, initiation of neural tube closure, neural crest cells migration, and correct formation of the heart. To study the neural tube closure phenotype, *Med12*$^{\Delta 1\text{-}7/wt}$;*CMV-Cre* heterozygous female embryos with mosaic expression of *Med12* were instrumental since they developed up to E17.5 and confirmed the NTDs in the absence of Med12. Additionally, all known examples of NTDs

were identified in these mutants: craniorachischisis, exencephaly and spina bifida thus identifying Med12 as a central regulator necessary for successful neural tube closure. Finally, the *Med12*$^{\Delta 1\text{-}7/Y}$;*Prx1-Cre* embryos showed that Med12 is involved in the early steps of chondrogenesis.

These different mutants allowed the identification of developmental processes controlled by Med12 and demonstrated that important signaling pathways and transcription factors require Med12 for their activity. The experiments shown in Chapter 2 demonstrated that the canonical Wnt/β-catenin signaling pathway uses Med12 for activation of its target genes and for regulation of processes such as EMT, body axis elongation or somitogenesis, all known to be regulated by the Wnt/β-catenin pathway. Additionally, non-canonical Wnt/PCP signaling is also disrupted in the absence of Med12 although it is still not possible to address the question of whether Med12 is an integrant part of the pathway or if it is necessary for expression of some of its core components.

This work highlights the importance in murine genetics of studying alleles with different expression levels of a targeted gene to allow a graded severity of phenotypes. This is particularly vital in cases where early embryonic lethality masks later developmental functions of the gene under study. This work is also a classical example of how the Cre-*loxP* system has revolutionized biology by providing a robust technique that allows quick generation of mammalian genetic models and that provides spatial and temporal control of gene ablation.

5.2 Questions arising

Although it could be unequivocally shown that several developmental processes regulated by canonical Wnt signaling use Med12 for integration of cell stimuli at the level of transcription, other known Wnt targets and processes seemed unperturbed or a fully affected “Wnt phenotype” failed to be observed. Examples of such observations were seen in practically all analyzed transgenic models.

Embryos totally depleted of Med12 (*Med12*$^{\Delta 1\text{-}7}$) died at gastrulation, and although EMT was deficient and primitive streak marker genes failed to be expressed, some cells were still able to delaminate of the epithelial epiblast layer and form a rudimentary mesoderm. This is in contrast to what has been described for mutants of the Wnt ligand Wnt3 or in mutants of the Wnt effector, β-catenin, where only two germ layers are built (ectoderm and primitive endoderm) and no delamination can be seen (Huelsken et al., 2000; Liu et al., 1999). In the hypomorphic mutants (*Med12*^{hypo}) a similar situation was observed. Mouse mutants for targets or components of the Wnt signaling pathway such as Wnt3a, T or the *β-catenin*^{floxdel}*;T-Cre* embryos all complete gastrulation, build practically no somites, have a generalized mesoderm formation deficiency, and produce an ectopic neural tube (Aulehla et al., 2008; Takada et al., 1994; Yamaguchi et al., 1999). The *Med12*^{hypo} embryos however, displayed severe somite patterning and differentiation defects but could built several somites. Ectopic neural tubes were also not observed in these embryos and additionally, the neural plate failed to close. Finally, expression analysis of known Wnt target genes in *Med12*^{hypo} embryos revealed that they had transcription affected mainly in the axial mesoderm while in lateral mesoderm or neural tissues expression was intact. The undisturbed limb patterning of *Med12*$^{\Delta 1\text{-}7}$*;Prx1-Cre* embryos is also not in line with disrupted Wnt signaling as it was shown that Fgf8 expression at the AER is controlled by Wnt signals (Barrow et al., 2003). Moreover, *Lmx1b* expression is regulated by the canonical Wnt ligand Wnt7a (Hill et al., 2006), and it is also correctly expressed in the absence of Med12.

In the case of incomplete Wnt phenotypes observed in *Med12*^{hypo} mutants it seems that the residual expression of Med12 (to less than 10% of wild-type levels), can still lead to Wnt transcriptional output since the phenotype is aggravated in embryos fully incapable of Med12 expression. The defective "epithelial" mesoderm observed in null *Med12*$^{\Delta 1\text{-}7}$ embryos suggests on the other hand, that some Wnt signaling occurs in the absence of Med12, similar to Wnt3a and T mutants (Liu et al., 1999; Yamaguchi et al., 1999), but is insufficient to induce complete EMT. This is further confirmed by the luciferase reporter experiments of Publication 1, where Med12 null cells were less responsive to Wnt ligands in comparison to their hypomorphic counterparts, but were still capable of inducing transcription. It seems then that on its absence, the role of Med12 as a coactivator for Wnt signaling might be performed by other proteins. Good candidates could be other subunits of the complex since it has been described for other transcription factors that more than one subunit can target the same TF to the Mediator (Uhlmann et al., 2007). Mediator-independent mechanisms for the activation of Wnt targets might also occur, which could explain some of the incomplete phenotypes described here, such as the correct patterning of mouse limbs that is usually disturbed in Wnt mutants.

Similarly, although experiments from Chapter 4 have shown that Med12 acts as a coactivator for Sox9 during embryonic skeletal development, the phenotype of *Med12*$^{\Delta 1\text{-}7}$*;Prx1-Cre* embryos was not as severe as it has been reported for embryos from matings of *Sox9*^{floxed} with *Prx1-Cre* mice. While *Sox9;Prx1-Cre* mutants displayed a total absence of skeletal structures, *Med12* hemizygous embryos could develop cartilage and bony elements especially in the hindlimbs. A potentially redundant role of Med12 in the coactivator function of Sox9 could in this case also be the cause of this observation. Additionally, the mixed genetic background of *Med12*^{flox} mice can lead to a modification of the phenotype. *Med12*^{flox} mice were generated by gene-targeting of G4 ES cells which are derived from F1 hybrid embryos from matings of two genetically distinct mouse strains: C57BL/6 and 129Sv (George et al., 2007). Intercrosses of these mice generate a very mixed genetic background. Occurrence of phenotype modifiers is

well documented in mouse genetics and many reports describe examples of phenotypes that are fully hidden or have reduced severity caused by the mixed genetic background (Haberland et al., 2009; Song et al., 1999). Such situation might be behind the differences between Med12 and Sox9 skeletal defects and it may also explain why Med12 absence allows some mesoderm formation while Wnt3- and β-catenin- deficient mice are deprived of this germ layer.

5.3 Project outlook

As aimed, transgenic mouse models have helped enormously to clarify the role of Med12 in transcriptional regulation during mouse development. In addition, the experiments described here raise new questions and also provide useful tools to expand studies concerning action of Med12 and the Mediator. *Med12*^{flox} ES cells and mice are excellent models to address such questions as they can provide tissue-specific gene inactivation.

Matings of *Med12*^{flox} homozygous females with male mice carrying Cre transgenes capable of tissue-specific expression will provide valuable information concerning new functions of Med12. Many interesting additional Cre-deleter lines are easily available that would help to clarify some of the phenotypes reported here. As mentioned in Chapter 4, the *Col2a1-Cre* mouse line (Ovchinnikov et al., 2000) provides recombination in all cells fated to become chondrocytes and therefore could show if in addition to specification of the chondrogenic lineage, Med12 is also necessary at later steps of skeletal development.

The failure of neural crest cell migration seen in *Med12*^{hypo} embryos is also of very high interest as these cells (also known as the fourth germ layer) contribute to several structures in the vertebrate body such as the cranial-facial skeleton and the peripheral nervous system. The *Wnt1-Cre* mouse line is invaluable for studies involving NCCs (Danielian et al., 1998) since it allows a precise and unique excision in these cells providing an excellent model to investigate the function of Med12 in skeletogenesis of the developing skull, and to examine a potential role in the differentiation of the nervous system.

Furthermore, other Cre-deleter lines can prove useful to individualize some of the different phenotypes observed in mutant embryos. Mesoderm induction and segmentation could be studied using the *T-Cre* mouse line that leads to gene ablation solely in mesodermal tissue, (Perantoni et al., 2005) thus avoiding interference in the phenotypic analysis from tissues such as the neuroectoderm. Reversibly, *Sox1-Cre* mice express the Cre recombinase in the neural

tube (Ovchinnikov et al., 2000) and in conjunction with *T-Cre* mice would help in studying potential interactions of these two structures that are mediated by Med12.

Some of the conclusions from this work provide novel views into biological processes controlled by Med12, which can be further investigated with some of the tools used throughout this thesis. The unexpected involvement of Med12 with Wnt/PCP signaling is one of the research topics that this thesis starts.

The PCP signaling pathway was first identified in *Drosophila melanogaster* and although its role during mouse development is only beginning to be understood, it has already been shown to control several developmental processes (Wang and Nathans, 2007). PCP is known to be essential for body axis elongation and a defective convergent extension of neuroectoderm cells is the most likely cause of the NTDs in *Med12* mutant embryos since elongation is required to provide the physical driving force for elevation of neural folds. In addition to the published NTDs described in Chapter 3, other PCP phenotypes were observed in *Med12*$^{\Delta 1\text{-}7/wt}$*;CMV-Cre* heterozygous females that are related with deficient migration of cells on a epithelial plane such as failure to close the eye lids and the presence of abdominal hernias later in development caused by an open body cavity (Yu et al., 2010).

All these phenotypes clearly show the involvement of Med12 in PCP signaling and the mislocalization of one of its core components (Prickle1) in *Med12*^{hypo} mutants supports this theory. Considering the role of Med12 as a transcriptional coregulator it is plausible that it functions together with a transcriptional downstream effector of the PCP pathway in a similar fashion to what is known for β-catenin in canonical Wnt signaling. The transcription factor c-Jun has already been implicated as being downstream of PCP signals although it was shown that it does not play a role in neural tube closure (Ybot-Gonzalez et al., 2007). This suggests that other TFs might perform such role and that Med12 could be a coregulator for their activity.

The precise molecular explanation for the PCP disturbance in *Med12* mutants gains then a special interest as, in addition to clarify how Med12 causes NTDs, it might also shed light into

transcriptional events controlled by this signaling pathway and concomitantly lead to identification of downstream effectors. For this, an essential tool will be the *Med12* heterozygous females described in Chapter 3 as these mice can survive up to E17.5 thus allowing the study of PCP in the sensory cells of the inner ear, a well described PCP controlled-process (Narimatsu et al., 2009).

The experiments from Chapter 2 have shown that Med12 not only binds to β-catenin but it is also required for activation of its target genes during mouse development in response to ligands of the canonical Wnt pathway. This is of crucial importance to human diseases such as colon cancer as it is known that aberrant activation of Wnt signaling leads to tumor initiation (Clevers, 2006; Fearon and Vogelstein, 1990).

Accordingly, recent reports have shown that Cdk8 (together with Med12, a component of the CDK8 module of the Mediator) is a potent oncogene required for the pernicious processes controlled by β-catenin (Firestein et al., 2008; Morris et al., 2008). *Med12*^{flox} mice can therefore be instrumental to test whether Med12 has an impact in such cancer events by serving (together with Cdk8) as a coactivator for β-catenin. A recent report has described a method that allows *in vitro* culture of mouse small intestine crypts that faithfully recreates how these structures function *in vivo* (Sato et al., 2009). Within intestinal crypts, stem cells are responsible for renewal of the intestine epithelium and mutations that disrupt their function are known to lead to tumor formation (Barker et al., 2007). Using this system, intestinal crypts from *Med12*^{flox} mice might reveal whether Med12 is required for the proliferation and differentiation of intestinal stem cells, since these processes are also controlled by canonical Wnt signaling. Additionally the system provides a platform to study a potential role of Med12 in tumor initiation. For this, excision of the floxed allele can be induced using the fusion protein Tat-Cre (Yu et al., 2003) that is added to cell culture medium and efficiently excises *Med12* in *Med12*^{flox} cells. Hopefully, such studies could show that interference of the Med12/β-catenin interaction is a potential target for colon cancer drug development.

6 Summary

The Mediator complex is commonly seen as a molecular bridge that connects DNA-bound transcription factors to the RNA polymerase II (Pol II) machinery. It is a large complex consisting of 30 subunits that is present in all eukaryotes. The Med12 subunit has been implicated not only in the regulation of Pol II activity, but also in the binding of transcription factors to the bulk of the Mediator complex.

To investigate its role during mouse development, the *Med12* gene was targeted in mouse embryonic stem cells (ES), producing a conditional null allele that expressed Med12 at wild-type levels. This allowed the generation of several mutant *Med12* alleles with varying amounts of Med12 expressivity and the analysis of mouse embryos carrying such alleles. These demonstrated that Med12 is essential for several processes of early mouse development such as mesoderm induction and segmentation, body axis elongation, formation of the heart and neural crest cell migration. Mice carrying the conditional allele were instrumental in the study of later developmental functions of Med12 like its involvement in skeletogenesis and neural tube closure.

Additionally, analysis of the phenotypes in mutant embryos established Med12 as a central component of important signaling pathways controlling mouse development. Specifically, results from this work showed that Med12 is critical for canonical Wnt signaling by acting as a coactivator for its nuclear effector, β-catenin. Further, the non-canonical Wnt/Planar Cell Polarity pathway also requires Med12 for its correct establishment during embryogenesis. And finally, experiments described here highlighted the importance of Med12 during induction of the chondrogenic lineage by acting as a coactivator for Sox9.

It is now clear that Med12 is an essential regulator of mouse development that coordinates several embryonic processes and is responsible for interpreting stimuli from signaling pathways by regulating transcription of their target genes.

7 Zusammenfassung

Der Mediatorkomplex gilt als eine molekulare Brücke zwischen an DNA-gebundenen Transkriptionsfaktoren und der RNA-Polymerase II Transkriptionsmaschinerie. Er besteht aus 30 Untereinheiten und findet sich in allen Eukaryonten. Die Med12-Untereinheit des Mediatorkomplexes spielt eine wichtige Rolle bei der Regulation der RNA-Polymerase II-Aktivität, sowie bei der Bindung von Transkriptionsfaktoren an den Komplex.

Um die Rolle von Med12 in der Mausentwicklung zu studieren, wurde das Med12-Gen in embryonalen Stammzellen (ES-Zellen) der Maus genetisch manipuliert. Hierbei wurden ES-Zelllinien mit verschiedenen Med12-Allelen generiert: ein konditionelles-, ein hypomorphes- und ein null-Allel. Das konditionelle Allel zeigte die gleiche Med12-Expression wie das Wildtyp-Allel. Die anderen Allele reduzieren die Med12-Expressionsstärke. Diese verschiedenen ES-Zelllinien wurden zur Generierung und zur Analyse von mutanten Mausembryonen verwendet, deren Phänotyp analysiert wurde. Die mutanten Mausembryonen zeigten, dass Med12 für unterschiedliche Prozesse der frühen Embryonalentwicklung, wie Mesoderminduktion, Segmentierung, Verlängerung der Körperachse, Herzbildung und Wanderung der Neuralleistenzellen essentiell ist. Mäuse, die das konditionelle Allel trugen, konnten für das Studium später Entwicklungsprozesse wie Skelettbildung und Schließung des Neuralrohrs verwendet werden.

Die Analyse der Phänotypen in Med12-mutanten Embryonen zeigte, dass Med12 als eine zentrale Komponente wichtiger Signaltransduktionswege die Mausentwicklung kontrolliert. Die Ergebnisse dieser Arbeit zeigen, dass Med12 als Koaktivator von β-catenin essentiell für den kanonischen Wnt-Signaltransduktionsweg ist. Der "Planar-Cell-Polarity Pathway", eine nicht-kanonische Variante des Wnt-Signaltransduktionswegs, ist während der Embryonalentwicklung ebenfalls auf Med12 angewiesen. Weiterhin konnte experimentell nachgewiesen werden, dass Med12 als Ko-Aktivator des Transriptionsfaktors Sox9 für die Induktion der Chondrogenese wichtig ist.

Zusammenfassend konnte innerhalb dieser Arbeit gezeigt werden, dass Med12 ein essentieller Regulator der Mausentwicklung ist, und dass Med12 durch Interpretation von Stimuli von Signaltransduktionswegen diverse embryonale Prozesse koordiniert und die Transkription verschiedener, entwicklungsrelevanter Zielgene reguliert wird.

8 Abreviations

AP	anterior posterior axis
AER	apical ectodermal ridge
AVE	anterior visceral endoderm
CE	convergent extension
CRS	craniorachischisis
CTD	carboxy terminal domain
DNA	desoxyribunucleic acid
DNMT	DNA methyltransferase
E	embryonic day
EMT	epithelial to mesenchymal transition
ES	embryonic stem
Fgf	fibroblast growth factor family
floxed	flanked by *loxP* sites
GTF	general transcription factor
HAT	histone acetyltransferase
HMT	histone methyltransferase
NCC	neural crest cell
NR	nuclear receptor
NTD	neural tube closure defect
PCP	planar cell polarity
PIC	pre-initiation complex
Pol	RNA polymerase
PS	primitive streak
RNA	ribonucleic acid
RT	room temperature
Shh	sonic hedgehog
TF	transcription factor
TSS	transcriptional start site
WISH	whole mount in situ hybridization
Wnt	wingless-type MMTV integration site family
XLMR	X-linked mental retardation
ZPA	Zone of polarizing activity

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10 Curriculum Vitae

For reasons of data protection, the Curriculum vitae is not published in the online version

11 List of Publications

- **Velica, P., Davies, N. J., Rocha, P. P., Schrewe, H., Ride, J. P. and Bunce, C. M.** (2009). Lack of functional and expression homology between human and mouse aldo-keto reductase 1C enzymes: implications for modelling human cancers. *Molecular Cancer* **8**, 1-12.

- **Rocha, P. P., Bleiss, W. and Schrewe, H.** (2010a). Mosaic expression of Med12 in female mice leads to exencephaly, spina bifida, and craniorachischisis. *Birth Defects Res A Clin Mol Teratol* **88**, 626-627.

- **Rocha, P. P., Scholze, M., Bleiss, W. and Schrewe, H.** (2010b). Med12 is essential for early mouse development and for canonical Wnt and Wnt/PCP signaling. *Development* **137**, 2723-2731.

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3. **Arnold, S. J. and Robertson, E. J.** (2009). Making a commitment: cell lineage allocation and axis patterning in the early mouse embryo. *Nat Rev Mol Cell Biol* **10**, 91-103.
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