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The regulation of *Dkk1* expression during embryonic development

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ABSTRACT

During embryogenesis, the Dkk1 mediated Wnt inhibition controls the spatiotemporal dynamics of cell fate determination, cell differentiation and cell death. Furthermore, the Dkk1 dose is critical for the normal Wnt homeostasis, as alteration of the Dkk1 activity is associated with various diseases. We investigated the regulation of *Dkk1* expression during embryonic development. We identified nine conserved non-coding elements (CNEs), located 3′ to the *Dkk1* locus. Analyses of the regulatory potential revealed that four of these CNEs in combination drive reporter expression very similar to *Dkk1* expression in several organs of transgenic embryos. We extended the knowledge of *Dkk1* expression during hypophysis, external genitalia and kidney development, suggesting so far to unexplored functions of *Dkk1* during the development of these organs. Characterization of the regulatory potential of four individual CNEs revealed that each of these promotes *Dkk1* expression in brain and kidney. In combination, two enhancers are responsible for expression in the pituitary and the genital tubercle. Furthermore, individual CNEs mediates craniofacial, optic cup and limb specific *Dkk1* regulation. Our study substantially improves the knowledge of *Dkk1* regulation during embryonic development and thus might be of high relevance for therapeutic approaches.

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Introduction

Wnt family proteins are involved in several developmental processes and diseases (Cadigan and Nusse, 1997; Moon et al., 2004). Canonically, the Wnt signaling pathway is transduced via two different transmembrane receptors, Frizzled- and Lipoprotein-receptor-related protein 5 and 6 (Lrp5/6). This signaling causes an inactivation of Gsk3β, resulting in a stabilization of β-catenin. Thereby, β-catenin enters the nucleus and promotes target gene expression by interaction with Tcf/Lef transcription factors (Baeg et al., 2001; Wehrli et al., 2001). The secreted and soluble cystein rich protein Dkk1 blocks canonical Wnt signaling by forming a ternary complex with Lrps and Kremen, a further transmembrane receptor. Thereby, a rapid endocytosis and removal of the Lrp receptor from the plasmamembrane is induced (Mao et al., 2002). Various embryonic patterning processes require a distinct Dkk1 mediated Wnt inhibition via this interaction.

The relevance of the Dkk1 mediated Wnt inhibition was initially demonstrated by Glinka et al. (1998) and Mukhopadhyay et al. (2001), who identified Dkk1 as a potent “head inducer” in metazoan development. Besides its essential function during head induction, *Dkk1* is expressed within various head derivates during later head development, such as the first branchial arch and its craniofacial derivatives, the brain and eyes (Monaghan et al., 1999; Nie, 2005; Nie et al., 2005; Lewis et al., 2007). These data suggest additional functions of *Dkk1* in a variety of developmental processes during head development.

During limb development, Dkk1 mediated inhibition of the canonical Wnt signaling is required for compaction of the apical ectodermal ridge (AER), a domain of epithelial cells at the distal tip of the limb bud that governs proximo-distal limb outgrowth (Grotewold et al., 1999; Church and Francis-West, 2002; Barrow et al., 2003; Soshnikova et al., 2003; Adamska et al., 2003). The expanded AER in *Dkk1*−/− mice results in polysyndactyly (Mukhopadhyay et al., 2001). In turn, overexpression of *Dkk1* in chicken limb buds induces programmed cell death in the AER, resulting in limb truncations (Grotewold and Rüther, 2002). To induce *Dkk1* transcription, Bmp4 activates the transcription factor c-Jun via signaling through the N-terminal c-Jun-kinase (Jnk) (Grotewold and Rüther, 2002). Recently, we have demonstrated that the teratogen thalidomide provokes limb defects by causing caspase-mediated cell death in early limb buds via perturbing the Bmp/Dkk1/Wnt pathway. This indicates the relevance of these signaling processes for correct limb development (Knobloch et al., 2007, 2008).

In the mouse mutant *doubleridge*, 60 kb localized 160 kb 3′of the last exon of *Dkk1* are deleted (Adamska et al., 2003). In these mice, *Dkk1* expression is dramatically reduced in head, limbs and tail, resulting in fore limb postaxial polysyndactyly (MacDonald et al., 2004; Adamska et al., 2004). Further reduction of Dkk1 by combining the *doubleridge* mutant with one *Dkk1* null allele resulted in additional head phenotypes, such as anophthalmia, hypoplastic anterior head structures and vertebral fusions (Adamska et al., 2004). These findings

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suggest the presence of *cis*-regulatory elements in the *doubleridge* region of the *Dkk1* locus.

The regulatory control of the Dkk1 level also impacts the homeostatic Wnt signaling range. When the Dkk1 level declines, embryopathies and diseases such as cancer can occur (reviewed in Niehrs, 2006). Therefore, the Dkk1/Wnt homeostasis is critical throughout an animal lifespan. For example, elevated Dkk1 serum levels of osteosarcoma patients are thought to play a key role in this pathogenesis and its attenuation by immunodepletion represents an auspicious strategy for therapy (Lee et al., 2007). The tumor suppressor p53 plays an important role in the regulation of cell cycle progression, differentiation and apoptosis (Levine, 1997). p53 binding to the *Dkk1* promoter causes *Dkk1* upregulation, suggesting that Dkk1 might mediate p53 tumor suppression by antagonizing the Wnt signaling pathway (Wang et al., 2000). *Dkk1* misregulation is also associated with neuronal degeneration of Alzheimer's brain, as the beta-amyloid peptide causes an p53-dependent induction of *Dkk1* (Caricasole et al., 2004). Thus, unraveling the control mechanisms of *Dkk1* expression will help to get closer insights into the molecular pathology of *Dkk1*-mediated diseases.

We analyzed the regulation of *Dkk1* expression, and identified putative conserved regulatory sequences at the *Dkk1* locus. In total nine CNEs are located 3′ of the last exon of *Dkk1*. Four of these are sufficient to mirror the endogenous *Dkk1* expression pattern. Furthermore, we were able to extend the knowledge of the published *Dkk1* expression profile during pituitary, kidney and external genitalia development. Further characterization of the CNEs revealed that *Dkk1* regulation is promoted by CNEs commonly during brain and metanephros development, in combination during pituitary and genital tubercle development and unique during craniofacial, limb and optic cup development.

Results

The mouse 1.8 kb Dkk1 promoter fragment is not sufficient to express a reporter in transgenic embryos

Previous studies in tissue culture cells had revealed that the human *DKK1* gene is directly activated by Wnts via TCF/Lef1 sites within a 2 kb *DKK1* promoter fragment (Chamorro et al., 2005; González-Sancho et al., 2005; Niida et al., 2004). Furthermore, p53 was shown to bind a p53 target site 2.1 kb upstream to the human *DKK1* start codon (Wang et al., 2000). These data suggested that these sites might have regulatory capabilities even during embryonic development.

Similarly, six putative TCF/Lef1 binding sites and a weak putative p53 response element are located 1.2 kb upstream to the mouse *Dkk1* start codon (Suppl. Fig. 1A, triangles). To test the regulatory ability of the *Dkk1* promoter during development, we cloned a fragment, spanning 1.8 kb upstream and 60 bp downstream to the *Dkk1* transcriptional start site in front of the bacterial *lacZ* gene (Suppl. Fig. 1A). Transient transgenic embryos were generated and assayed for X-Gal staining at E12.5, a stage, at which *Dkk1* has previously been shown to be expressed in various tissues (Monaghan et al., 1999; Nie, 2005). None of the seven transgenic embryos displayed any reporter activity, as revealed by whole-mount stainings in comparison to the endogenous pattern (data not shown; Suppl. Figs. 1B and C). Thus, the 1.8 kb *Dkk1* promoter fragment is not sufficient for transcriptional activation of the *Dkk1* reporter construct in transgenic embryos.

Identification of nine conserved non-coding elements at vertebrate Dkk1 loci

Because the *Dkk1* expression pattern is evolutionary conserved during vertebrate embryonic development (Monaghan et al., 1999), we assumed that the transcriptional regulation of *Dkk1* might also be conserved. Therefore, we tried to identify conserved non-coding elements (CNEs) by *in silico* analyses of the mouse *Dkk1* locus (Fig. 1). A comparison between mouse and human *Dkk1* revealed several broad sequence blocks conserved between human and mouse at the *Dkk1* locus. A comparison with more ancestral mammalian genomes (e.g. *Monodelphis domestica*) revealed a reduced number of putative regulatory sequences. A comparison with the chicken *Dkk1* locus revealed that nine CNEs were already present at the *Carboniferous* period, located in a distance of 25 kb to 195 kb 3′ to the mouse *Dkk1* transcriptional start (Fig. 1). Using whole genome shotgun searches, we found that three of the CNEs were conserved at the *Dkk1* locus of the cartilaginous fish *Callorhinchus milii*, which arose 455 million years ago. The identified CNEs share no similarities, neither among each other, nor to conserved sequences at the *Dkk2* or *Dkk3* loci (data not shown). Therefore, we suppose that the identified CNEs evolved in parallel and are unique.

The identified CNEs are between 104 and 412 bp in length and each of them shares a sequence identity above 70% from chicken to human (Figs. 2B and A). Four CNEs are located inside the *doubleridge* location (Fig. 2A). Weakening the search criteria to 60% or 50% sequence identity increased the amount of putative regulatory sequences (data not shown). To analyze the regulatory potential of these CNEs, we cloned all or different combinations of CNEs, including 5′ and 3′ flanking sequences downstream of the SV40pA into the *Dkk1* promoter containing lacZ reporter construct (Fig. 2C). The identified CNEs 190 and 195 have been cloned together as a 5.7 kb genomic fragment. A summary of constructs used in this study is given in Fig. 2C.

Four CNEs activate the reporter very similar to the endogenous Dkk1 expression in transient transgenic embryos

Transient transgenic embryos were generated and analyzed for reporter activity at E12.5, a stage at which *Dkk1* is characteristically expressed in craniofacial derivates, limb buds and optic cups (Figs. 3J–L; Monaghan et al., 1999; Nie, 2005). We initially tested three different

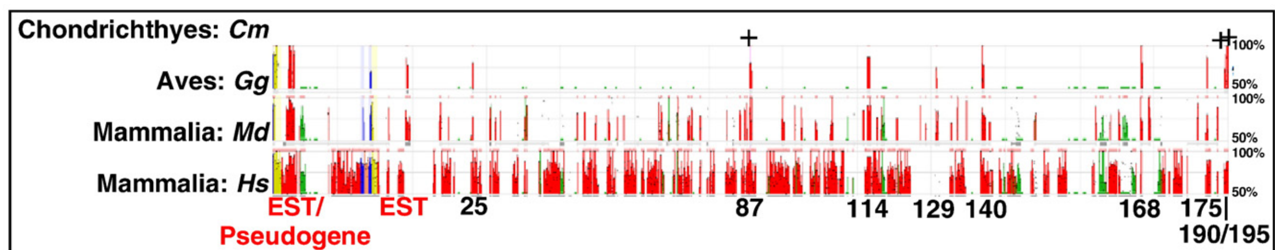
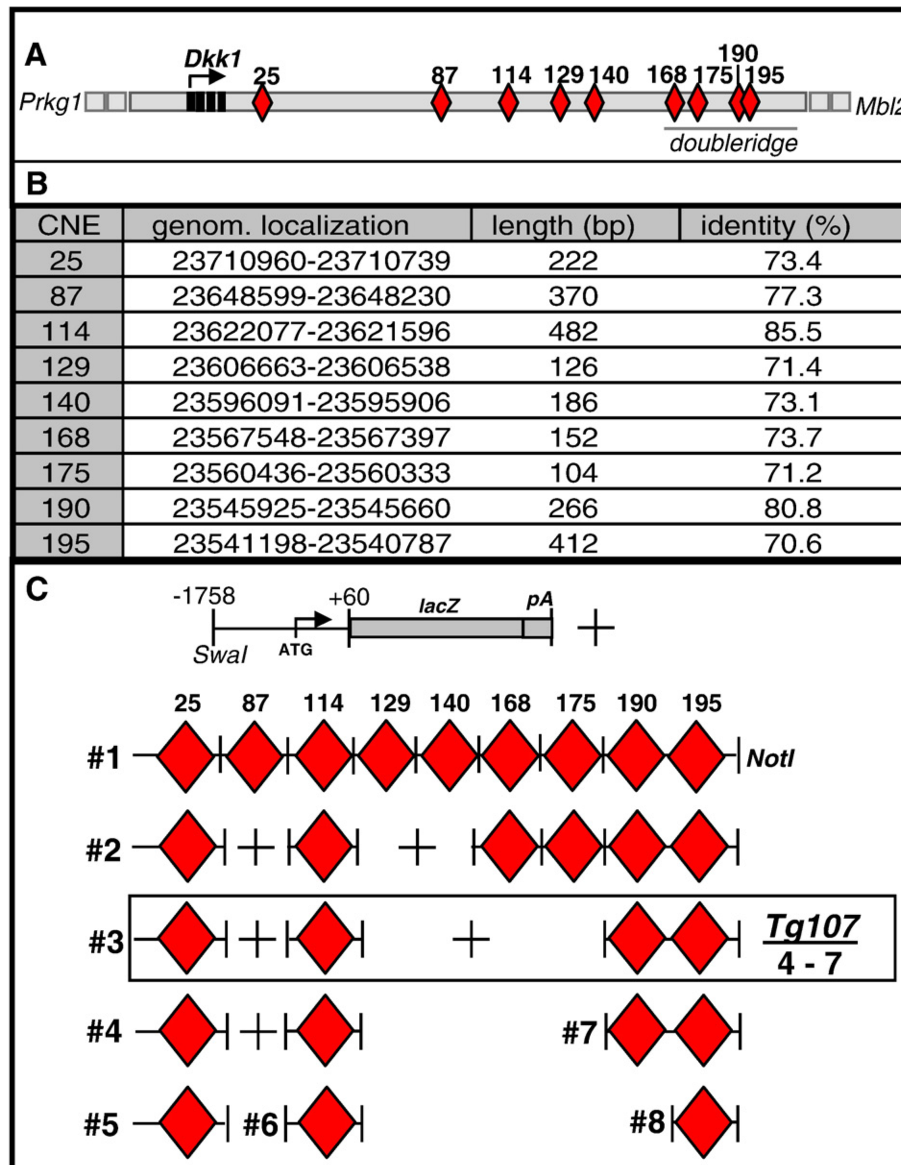


Fig. 1. Conserved synteny of *Dkk1* loci across vertebrates. The graph depicts *Dkk1* loci in cartilaginous fish (Chondrichthyes), bird (Aves) and mammalian (modified after "Ecr browser"). The mouse *Dkk1* locus was used as a basis. Red peaks imply newly identified CNEs. Nine of these sequences exist in the bird genome and are marked by numbers relative to the *Dkk1* transcriptional start in kb. Two conserved regions were identified in the cartilaginous fish genome by "whole genome shotgun" alignments and are indicated by (+). Repetitive elements (green), expressed sequence tags (ESTs) and pseudogenes were ignored in our analyses. Blue = *Dkk1* exons; yellow = *Dkk1* 3′UTR; green = transposons and simple repeats. Abbreviations: *Hs* = *Homo sapiens*; *Md* = *Monodelphis domestica*; *Gg* = *Gallus gallus*; *Cm* = *Callorhinchus milii*. Parameter settings (Ecr browser): minimal alignment score: 75%; length: 100 bp.



CNE	genom. localization	length (bp)	identity (%)
25	23710960-23710739	222	73.4
87	23648599-23648230	370	77.3
114	23622077-23621596	482	85.5
129	23606663-23606538	126	71.4
140	23596091-23595906	186	73.1
168	23567548-23567397	152	73.7
175	23560436-23560333	104	71.2
190	23545925-23545660	266	80.8
195	23541198-23540787	412	70.6

Fig. 2. Genomic organization of newly identified CNEs at the mouse *Dkk1* locus. (A and B) Location and characteristics of newly identified CNEs at the *Dkk1* locus. CNEs and their distances to the *Dkk1* start codon at 23735764 are indicated by red diamonds. Coordinates are in orientation to the *Dkk1* start, length in basepairs (bp); sequence identity between chicken and human are indicated. Note that four CNEs are located within the *doubleridge* location. (C) Scheme of constructs tested in this study. CNEs used for the generation of the transgenic mouse line (*Tg107*) are indicated.

combinations of CNEs in parallel to assess their regulatory ability. Embryos carrying construct #1 exhibited reporter gene activity at known *Dkk1* expression sites such as maxillar and mandibular regions, frontonasal process, optic cups and limb buds (Figs. 3A–C; compare to Grotewold et al., 1999; Monaghan et al., 1999; Nie, 2005). However, this transgene exhibited additional activity in dorsal head structures, as well as varying activity in the telencephalon and mesodermal structures and lateral to the developing spine. These data suggest that the tested construct contains sequences, which cause an ectopic reporter activity. Transgenic embryos carrying construct #2 showed activity in the same *Dkk1* specific expression domains (Figs. 3D–F). In the head, however, artificial reporter activity was now only detectable in the lateral telencephalon. All four transgenic embryos carrying construct #3 showed an almost identical reporter pattern in the brain, optic cups, craniofacial mesenchyme and limbs (Figs. 3G–I). These data suggest that CNEs 25, 114, 190 and 195 together function as *Dkk1* enhancers in a broad regulatory fashion.

Dkk1 expression is mainly regulated by four CNEs

Because all transient expressing embryos, carrying CNEs 25, 114, 190 and 195 exhibited an identical and *Dkk1* specific expression at E11.5 and E12.5 (data not shown; Figs. 3G–I), we used these CNEs for the generation of a transgenic mouse line (*Tg107*). As it has been described for the endogenous *Dkk1* expression the reporter is activated in the anterior head process and in the anterior mesendoderm at E7.5 (Fig. 4A; compare to Kemp et al., 2005; Barrantes et al., 2003). Reporter activity was detectable in the developing ventral midbrain, in the optic vesicles, the first branchial arch, the AER, the posterior necrotic zone (PNZ) and in the very distal mesenchyme of limb buds, closely resembling the *Dkk1* expression pattern at E10.5 (Figs. 4B and C; data not shown). At E12.5, reporter activity in derivates of the 1st branchial arch, in the optic cups and limbs was very similar to the endogenous *Dkk1* expression pattern (Fig. 4D). At later embryonic development, reporter activity is stable and in

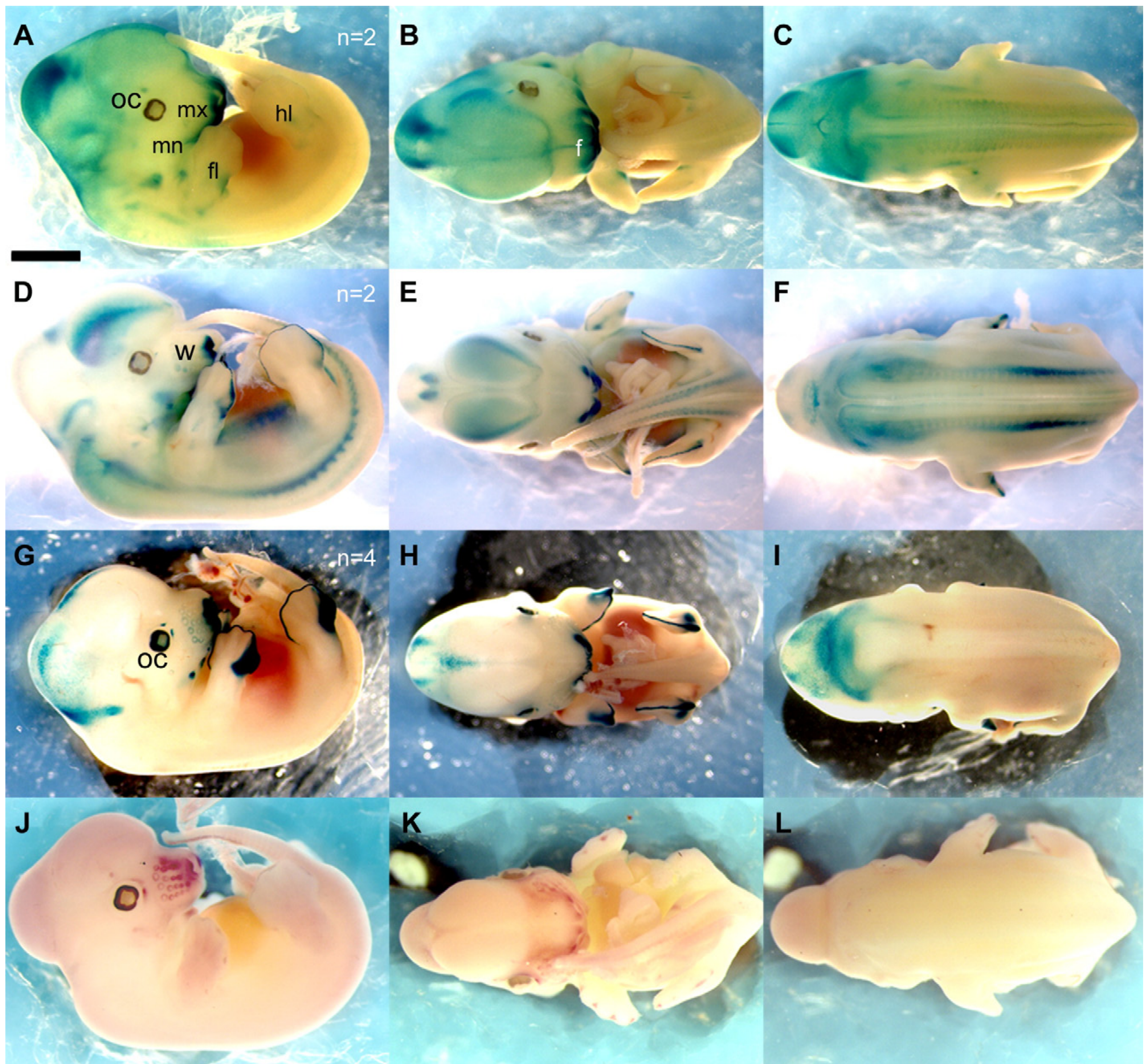


Fig. 3. CNEs 25, 114, 190 and 195 together promote reporter expression very similar to the endogenous *Dkk1* expression pattern. *LacZ* activity in whole-mount embryos at E12.5, under control of different combinations of CNEs tested in parallel. (A; D; G) Lateral views, (B; E; H) ventral views and (C; F; I) dorsal views of whole-mount lacZ stained embryos. (J–L) *Dkk1* expression pattern at E12.5 as a comparison. (A–C) All CNEs together (construct #1) mediate reporter activity in craniofacial maxillar (mx) and mandibular (mn) mesenchyme, fore limbs (fl), hind limbs (hl) and optic cups (oc). Additionally, the reporter is ectopically activated in the dorsal head and lateral to the spine. (D–F) CNE25, 114, 168, 175, 190 and 195 (construct #2) together promote activity in these domains; however, reporter activity in the head is more distinct. (G–I) CNE25; 114; 190 and 195 (construct #3) promote reporter expression in the eyes, craniofacial, limbs and brain, very similar to the endogenous *Dkk1* expression pattern (J–L). (A) Scale bar: 2 mm.

addition, it becomes active in the osteogenic centers of the bones (Fig. 4E). Given that the reporter activity of the mouse line is very similar to the *Dkk1* expression in ecto-meso and endodermal tissues, we focused on the regulatory properties of this line more in detail.

Regulation of Dkk1 expression in the neuroectoderm and pituitary

Dkk1 is dynamically expressed during neuroectodermal development (Monaghan et al., 1999; Diep et al., 2004). We therefore analyzed the neuroectodermal reporter activity and found expression in the ventral diencephalon at E9.5 adjacent to the Rathke's pouch (Fig. 5A). After ectodermal invagination towards the 3rd ventricle, reporter expression increases (Fig. 5B). Reporter activity was detectable in the dorso medial telencephalon, the ventral thalamus and in the dorsal mesencephalon at E11.5 (Fig. 5C). *Dkk1* specific activation in the delaminating 3rd ventricle extends into the infundibulum of the hypophysis at this stage (Fig. 5D; *Dkk1* expression see inlay). Accordingly, *Dkk1* is expressed in the pituitary during *Xenopus* development (Monaghan et al., 1999). At E12.5, *Dkk1* specific activation in mesencephalon increases and also appears in the rhombencephalon (Figs. 5E and F; comparison between the reporter activity and *Dkk1* expression see inlays and F). Activation in the hypophysis exclusively occurs within the posterior part and not within the Rathke's pouch, as revealed by transverse sections (Fig. 5G). At E14.5, ventral expression domains in the diencephalon and pituitary are no longer detectable, while dorsal mesencephalic and rhombencephalic expressions are maintained (Fig. 5H). Besides these expression domains, we found characteristic reporter activity in the ventral neural tube and lateral to it (Figs. 5I and J). These data demonstrate that reporter gene expression is very similar to the endogenous *Dkk1* expression pattern

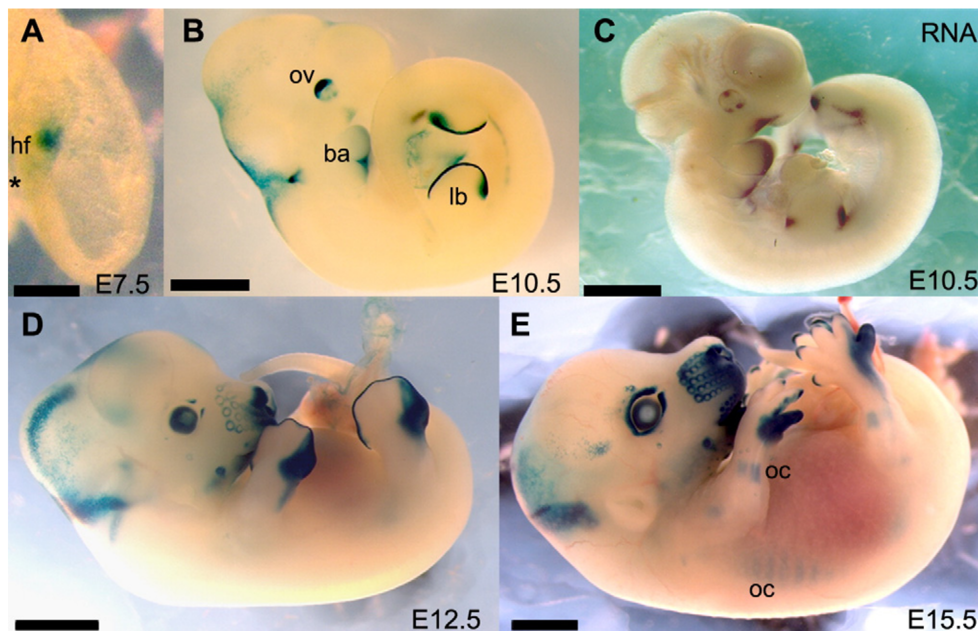


Fig. 4. The *Tg107* line closely resembles the endogenous *Dkk1* expression. (A,B,D,E) Sideviews of β-Gal stained embryos, in comparison to the endogenous *Dkk1* expression (C). Developmental stages are indicated. (A) Activity in the anterior head fold (hf) and the anterior mesendoderm (*) at E7.5. (B) Reporter activity in several head derivates, such as optic vesicles (ov), the 1st branchial arch (ba) and limb buds (lb), in comparison to *Dkk1* expression at E10.5 (C). (D) Expression appears in 1st branchial arch derivates, such as whisker hair bud mesenchyme and characteristically in the eyes, eyelids and limbs at E12.5. (E) Reporter activity is stable and additionally detectable in the osteogenic center (oc) of the developing bones at E15.5. Scale bar: 200 μm in (A); 400 μm in (B,C); 2 mm in (D,E).

during neuroectodermal development. Therefore, these data suggest that *Dkk1* might be involved in hypophysis development.

Regulation of craniofacial Dkk1 expression

As the reporter activity in the *Tg107* mouse line is identical to the *Dkk1* expression during head induction and later in the 1st branchial arch (Fig.4), we analyzed the regulatory potential of the CNEs during craniofacial development in detail. Besides lacZ activity in the optic vesicles, the reporter is active in the distal mandibular mesenchyme of the 1st branchial arch at E9.5 (Fig. 6A). At E10.5, reporter activity is additionally evident in the distal maxillar region (Fig. 6B). Reporter activity appears in the medial mesenchyme of the 1st branchial arch and the border to the 2nd branchial arch (Fig. 6C). Maxillar reporter activity intensifies until E11.5 (Fig. 6D). Additionally, lacZ is detectable in the lateral, but not in the frontonasal process (Fig. 6E). At E12.0, *lacZ* expression is activated in the frontonasal process and whisker hair bud mesenchyme (Fig. 6F). Until E12.5, reporter activity in these domains is stable and is detectable additionally in the distal tongue (Figs. 6G and H). Note, that in contrast to the endogenous *Dkk1* expression in the mesenchyme below the vomeronasal domain, the reporter is not activated in this domain (Figs. 6H and I in comparison to J). However, expression in the mesenchyme of the vomeronasal organ requires at least CNEs 168 and/or175 (Suppl. Fig. 2). Beside this, reporter activity is *Dkk1* specific in the tooth bud mesenchyme and weak in the palatal ectoderm (Fig. 6K). Furthermore, reporter activity is evident in the whisker hair bud mesenchyme but not ectoderm (Fig. 5L). Craniofacial activity is stable at later craniofacial developmental stages (Figs. 6M and N). Thus, during craniofacial development, reporter activity in *Tg107* embryos is very similar to the published endogenous *Dkk1* expression data (Monaghan et al., Nie et al., 2005; Nie, 2005).

The regulation of Dkk1 expression during limb development

At E11.5, the *Tg107* reporter is active in the AER and additionally in a posterior mesenchymal domain, which is not *Dkk1* specific (compare Fig. 7A with Fig. 7D). At E12.5, reporter activity in the posterior mesenchyme is maintained, however not activated in the interdigital mesenchymal domain, as expected by the endogenous *Dkk1* expression pattern (Figs. 7B and E). After AER degeneration, reporter gene expression is maintained in the distal tip of the digits as described for endogenous *Dkk1* expression, however activity in the 5th digit is also maintained (Figs. 7C and F; Monaghan et al., 1999; Grotewold et al., 1999).

Regulation of Dkk1 expression during kidney and external genitalia development

Recent studies revealed that *Dkk1* is dynamically expressed in the posterior embryo and plays a role during embryonic segmentation (Aulehla et al., 2008). We found weak reporter activity in gastrulating mesodermal cells at E7.5 (Fig. 4A). Afterwards, mesodermal cells exhibit characteristic activity domains. When embryonic segmentation takes place, the reporter was activated in the posterior most somites and less intensive in the posterior presomitic mesoderm as well as in the closing neural tube (Fig. 8A). After embryonic turning, reporter gene activity characteristically remained in the most posterior somites and the posterior neural tube (Fig. 8B). At E9.5, activation was no longer detectable in the posterior somites and in the posterior neural tube (Fig. 8C; data not shown).

Next, we analyzed the spatiotemporal dynamics of the *Tg107* reporter activity during mouse urogenital development. Activation was specifically detected in intermediate mesodermal cells that have aggregated in a posterior region to form the pronephros at E9.5 (Figs. 8C and I). Furthermore, activation was observed in the ventral mesodermal region, reaching from the urogenital sinus to the tail bud. This domain resembles *Dkk1* expression in *Xenopus* embryos (Monaghan et al., 1999). Both the reporter gene and *Dkk1* are expressed in the nephric duct and in the mesonephros at E10.5 (Figs. 8D and E). Within the metanephros, reporter activity is detectable in the urether, in the buds and in the renal vesicles, very similar to the endogenous *Dkk1* expression pattern at E12.5 (Figs. 8F–H; Potter et al., 2007; Monaghan et al., 1999). At later kidney development, the

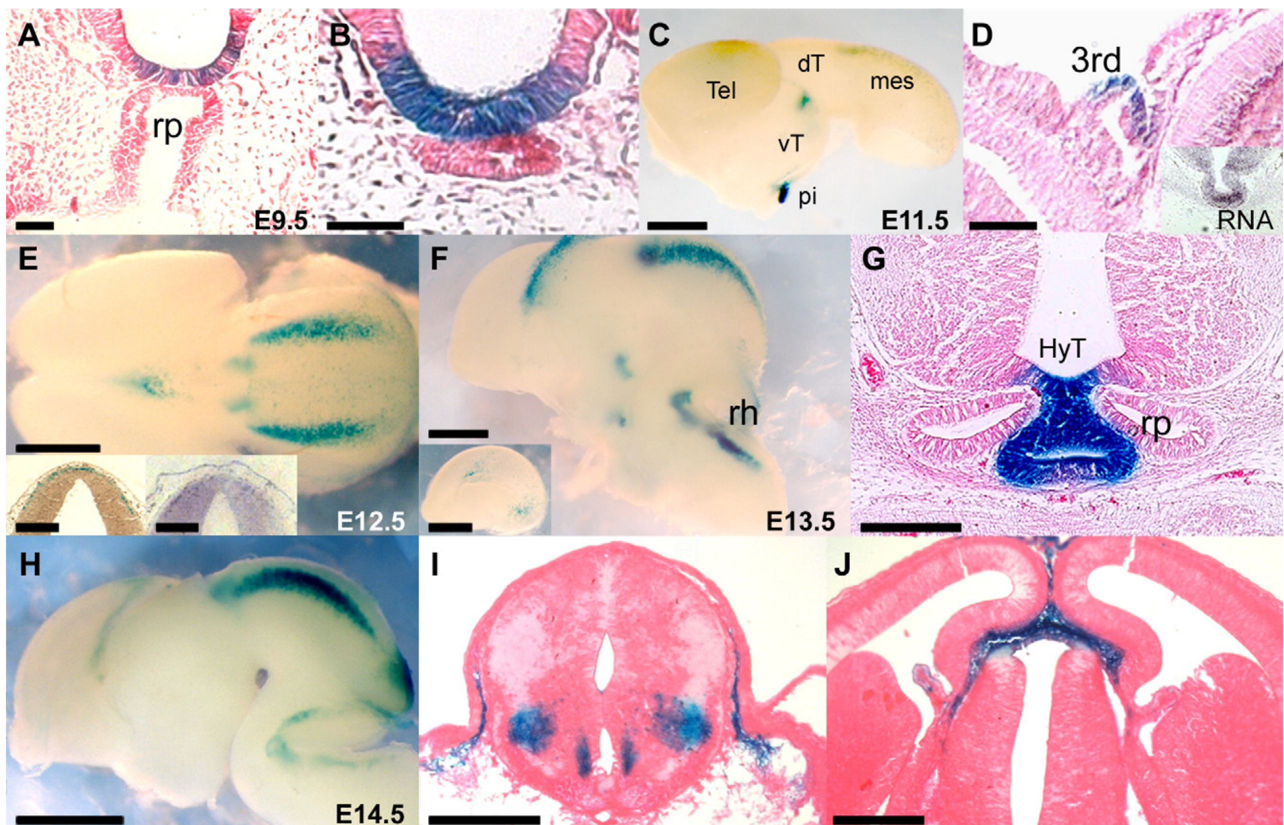


Fig. 5. Reporter expression in neuroectodermal derivates and the pituitary. (A–J) *LacZ* expression in neuroectodermal derivates. (A; B; G; I; J) Transverse sections; (D) sagittal section; (C; F; H) sideviews; (E) dorsal view. (A; B) Reporter activity increases in the ventral midbrain during rathke's pouch (rp) invagination at E9.5. (C) *LacZ* expression is detectable in the medial telencephalon (Tel), ventral thalamus (vT) and mesencephalon (mes) at E11.5. No expression appears in the dorsal thalamus (dT). (D) *LacZ* expression is evident in the region of the 3rd ventricle (*Dkk1* RNA, see inlay), delaminating into the posterior hypophysis. (E) Reporter expression is increased in dorsal neuroectodermal expression domains at E12.5 (*Dkk1* RNA, see inlay). (F) Characteristic expression in the telencephalon at E13.5 (see inlay), additionally in the rhombencephalon (rh). (G) At this stage, reporter activity in the ventral diencephalon is decreased, but still evident in the hypothalamus (HyT). Note, that expression does not extend to the rp. (H) Reporter expression in ventral diencephalon and the pituitary is no longer evident at E14.5. (I) Beside the reporter activity in the neuroectoderm in the head anlage, the reporter is evident in the ventral neural tube and dorsal root ganglia. (J) Reporter expression is also detectable in the medial mesenchyme between the telencephalic hemispheres at E14.5. Scale bar: 50 µm in (A,B); 200 µm in (inlays of E; D,G,I,J); 1 mm in (C,E,F); 2 mm in (H).

reporter is activated as previously described, in S-shaped and comma shaped bodies (data not shown; Monaghan et al., 1999).

We also found reporter activity in the urogenital sinus, specifically in the cloacal membrane from E9.5 onward (Figs. 8C and I). Therefore, we looked for the dynamics of reporter activation during external genitalia development. Transgene activity was found in the distal urethral epithelium and the adjacent mesoderm, resembling endogenous *Dkk1* expression at E11.5 (Figs. 8J and K). Activity is detectable in the distal urethral epithelium and lateral to the ventral midline at E12.5 (Fig. 8L). At E14.5, the reporter was active in the distal epithelium and lateral to the ventral midline (Fig. 8M). When the external genitalia were differentiated, reporter activity decreases, and is hardly detectable in males and females at E16.5 (Figs. 8N and O). At later stages, reporter activity was no longer detectable (data not shown). The above shown data reveal that *Dkk1* is expressed during kidney development earlier as described. Furthermore, we assume that beside the canonical Wnt signaling itself, Dkk1 might play a role during external genitalia development.

CNE114 promotes regulation of craniofacial Dkk1 expression

The reporter activity of the *Tg107* mouse line revealed that CNEs 25, 114, 190 and 195 in combination are sufficient to promote the regulation of *Dkk1* expression in most aspects. Therefore, we analyzed the regulatory properties and relationship of these putative enhancers individually. We cloned CNE25 and 114 together in the promoter reporter construct (#4) and analyzed transgene activity at E12.5. These CNEs directed reporter gene activity in the dorsal mesencephalon, the metanephros and in distal craniofacial domains, such as whisker hair bud mesenchyme and the mandibular and maxillary mesenchyme (Fig. 9A). We then generated transgenic mice, carrying CNE25 or CNE114 individually (constructs #5 and #6). Both CNEs alone mediated a similar lacZ staining in the brain and metanephros (Figs. 9B and C and data not shown). Furthermore, CNE114 alone mediated reporter expression additionally in craniofacial mesoderm, identical to the *Tg107* line (Figs. 9C and D). However, CNE114 does not mediate the vomeronasal mesenchymal activity (Fig. 9E), as suggested already by the analyses of the *Tg107* line (compare with Fig. 6). Taken together, the enhancer CNE114 promotes reporter activation during craniofacial development.

CNE195 mediates Dkk1 optic cup and AER Dkk1 regulation

In order to analyze the regulatory properties of CNEs 190 and 195, we cloned a 5.7 kb genomic fragment, containing both CNEs but none of the others into the *Dkk1*-promoter/lacZ reporter construct (#7). Besides brain and metanephros, this combination results in reporter activation in the optic cup and the developing limb buds (Fig. 9F; data not shown). Therefore, we tested these CNEs individually. CNE190 mediated reporter expression identical to CNE25 exclusively in the mid brain and metanephros (data not shown). CNE195 in a single construct (#8) mediated reporter activation in the anterior and central retina of the optic cup and the AER of the limb buds, suggesting that

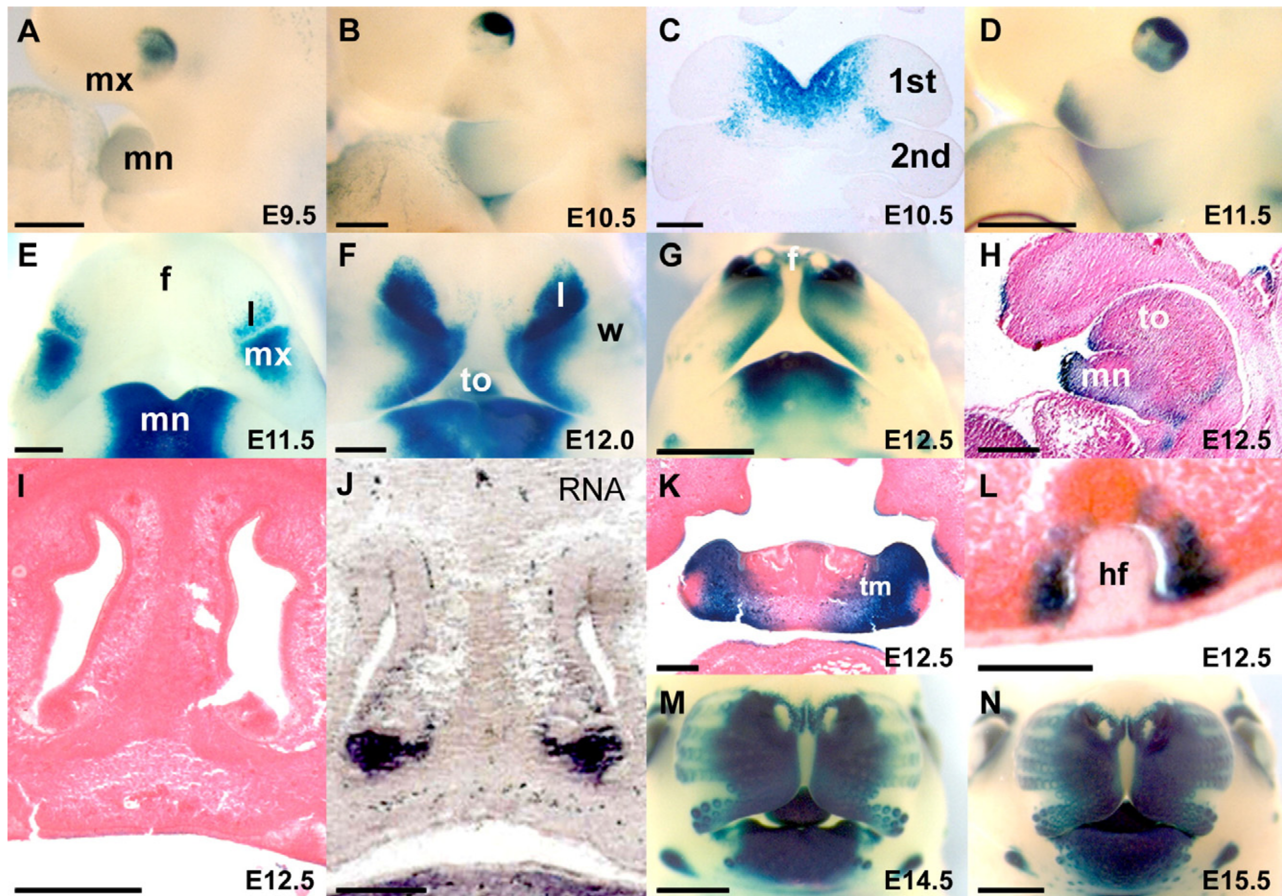


Fig. 6. Craniofacial *Dkk1* regulation. (A, B D, E, F, G, M, N) β-Gal activity in whole-mount embryos, on transverse (C, I, K and L) and sagittal sections (H); (J) endogenous *Dkk1* expression in the vomeronasal organ. (A) Activity appears in the mandibular mesenchyme (mn) of the 1st branchial arch at E9.5. No activity is visible in the maxillar mesenchyme (mx). (B) Reporter expression in the mx at E10.5. (C) Reporter activity is detectable in the medial mn mesenchyme. (D) Activity intensifies in the maxillar mesenchyme at E11.5. (E) Reporter activity is additionally detectable in the lateral but not frontonasal pit at this stage. (F) Expression is additionally visible in the frontonasal mass (f) and in the whisker hair buds (w) at E12.0. Reporter activity appears additionally in the tongue (to). (G) In these domains, expression is more intense at E12.5. The reporter is not activated in the mesenchyme below the vomeronasal domain, a region *Dkk1* expression is evident (H and I in comparison to J). (K) Reporter expression is detectable in the tooth mesenchyme (tm) of the mandible at E12.5. At the whisker hair follicle (hf), expression is detectable in the hair bud mesenchyme but not ectoderm. (M and N) Reporter activity is stable in craniofacial domains at E14.5 and E15.5. Scale bars: 100 μm in (C); 200 μm in (A,B,I–K); 250 μm in (E); 500 μm in (H,F); 1 mm in (G,M,N).

this sequence functions as a competent *Dkk1* eye and limb enhancer (Figs. 9G–I).

Discussion

Dkk1 CNEs communicate with the endogenous Dkk1 promoter

Previous data revealed that *Dkk1* transcription can be activated by Wnt targeting of Tcf/Lef sites located in the *Dkk1* promoter in vitro (Chamorro et al., 2005; González-Sancho et al., 2005; Niida et al., 2004). We found that the mouse *Dkk1* promoter, including these Tcf/Lef sites alone is not sufficient for activation of *Dkk1* expression at E12.5 (Suppl. Fig. 1). By sequence analyses, we found that neither the Tcf/Lef sites nor the p53 site, located in the *Dkk1* promoter are evolutionary conserved regions (data not shown). Thus, tissue specific activation of *Dkk1* expression during embryonic development requires additional regulatory sequences. Vice versa, we found that *Dkk1* CNEs cause a partially and generally weak activation of a β-globin minimal promoter (data not shown) and thus tissue specific *Dkk1* enhancer function requires the endogenous *Dkk1* core promoter. We assume that the endogenous *Dkk1* promoter is required to form the correct chromatin structure to allow interactions with the *Dkk1* CNEs. Similar results have recently been described for *engrailed* enhancers, which require the endogenous *engrailed* promoter for proper enhancer functioning during *Drosophila* development (Kwon et al., 2009).

Dkk1 transcriptional regulation is evolutionary conserved

We isolated nine CNEs, located 3′ to the *Dkk1* coding sequence at a distance from 25 kb to 195 kb to the mouse *Dkk1* transcriptional start. Three of these sequences, CNEs 87, 190 and 195 are deeply conserved, as we identified homologous regions in *Chondrichthyes* (compare with Suppl. Fig. 3). Four CNEs, 25, 114, 190 and 195 together promote reporter expression in a transgenic mouse line, very similar to the *Dkk1* expression pattern during embryonic development. We further characterized the regulatory potential of these CNEs (summarized in Table 1). All four CNEs have a common regulatory ability during brain and metanephros development, suggesting a multiple safety mechanism to ensure a minimal *Dkk1* expression level. Furthermore, we found a 5.7 kb sequence (containing CNE190 and 195), which promotes reporter activation in the hypophysis and the genital tubercle. CNE114 alone mediates activation in the craniofacial mesoderm and CNE195 functions as an enhancer in the optic cups and the limbs.

CNE195 mediated regulation of Dkk1 expression in the optic cups and limbs

Dkk1 is dynamically expressed during eye development (Monaghan et al., 1999; Ang et al., 2004). We found that the *Tg107* reporter is expressed identical to the endogenous *Dkk1* expression pattern in the optic cups. Furthermore, CNE195 alone promotes reporter activity in eye expression domains. Sequence analyses revealed that conserved

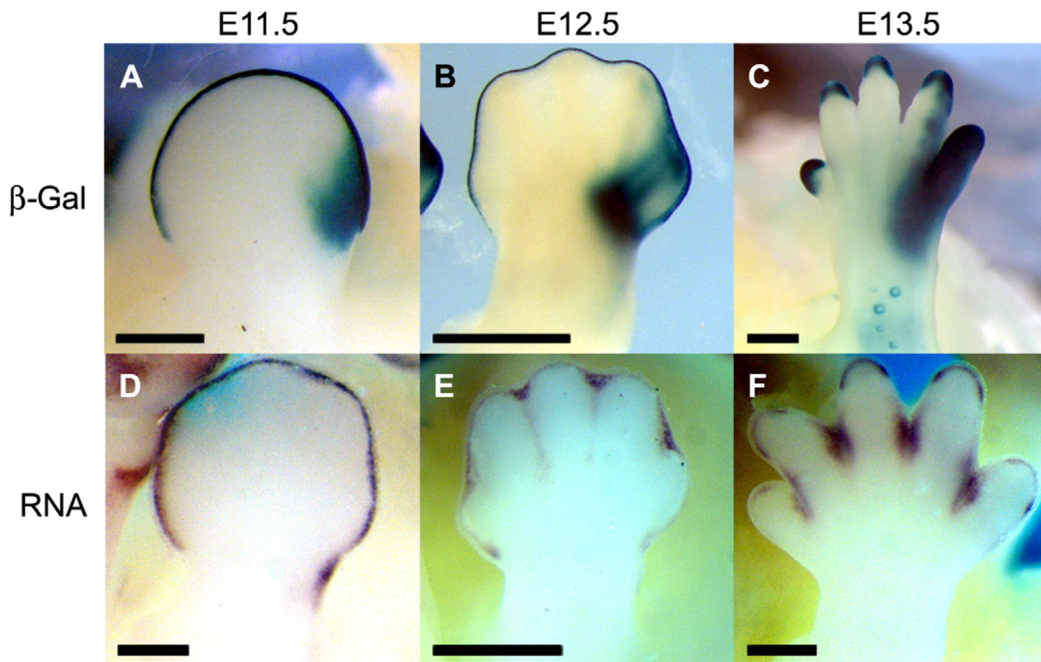


Fig. 7. The regulation of *Dkk1* expression during limb development. (A–C) β-Gal activity in comparison to the endogenous *Dkk1* expression (D–F). Stages are indicated; dorsal views, anterior is to the left. (A and D) β-Gal activity is comparable to the *Dkk1* expression in the AER but differing in the posterior mesenchyme at E11.5. (B and E) Reporter activity is found distally and in the posterior mesenchyme; interdigital *lacZ* expression fails in the transgene at E12.5. (C and F) Reporter gene activity marks the fifth digit and the posterior digit IV; interdigital expression is not evident at E13.5. Scale bar: 500 μm in (A,D,C,F); 1 mm in (B,E).

Tcf/Lef, Pax2 and Msx2 transcription factor binding sites are located within CNE195 (Suppl. Fig. 3). As these factors have essential functions during eye development and are at least partially coexpressed with *Dkk1* (Liu et al., 2003; Torres et al., 1996; Wu et al., 2003), they might facilitate *Dkk1* transcriptional activation by the direct binding to CNE195.

CNE195 also promotes reporter activation in the AER. This might be governed by direct targeting of CNE195 via a conserved Tcf/Lef site by the canonical Wnt signaling. In line with this suggestion is the fact that stabilization of β-catenin results in truncated limbs caused by a premature regression of the AER, similar to *Dkk1* overexpression in the limb (Hill et al., 2006 compare with Grotewold and Rüther, 2002). Beside this, evolutionary conserved transcription factor binding sites of Dlx5/6 and Msx2 are located in a conserved block within CNE 195 (Suppl. Fig. 3). As these genes are coexpressed with *Dkk1* within the AER, they might directly promote *Dkk1* activation via CNE195 (Kraus and Lufkin, 2006). Surprisingly, we could not identify any CNEs that promote *Dkk1* expression in the interdigital regions of the developing limbs. This result suggests that AER specific *Dkk1* expression during proximo-distal limb outgrowth is evolutionary more conserved than interdigital. Gene expression- and interdigital cell death varies in tetrapods and may therefore be regulated by a different mechanism. Further experiments will have to clarify the regulation of *Dkk1* expression during limb development.

CNE114 mediated regulation of craniofacial Dkk1 expression

The *Tg107* mouse line reflects mesodermal *Dkk1* activation within the 1st branchial arch, and later on in its mesodermal derivates such as craniofacial structures. A Wnt/Lef signaling reporter mouse line exhibits an identical activity within the 1st branchial arch, which later becomes downregulated in the craniofacial mesoderm (Mani et al., 2009). As CNE114 promotes mesodermal craniofacial activation of *Dkk1* expression and contains two conserved Lef1 binding sites, we suppose that activation might be mediated by direct targeting of CNE114 by the canonical Wnt signaling (Suppl. Fig. 3). *Dlx* genes are expressed in the first branchial arch and mutations in *Dlx*5/6 genes cause severe craniofacial phenotypes (Qiu et al., 1997; Robledo et al., 2002). Dlx transcription factors have conserved binding sites located within the CNE114 (Suppl. Fig. 3). Thus, Dlx proteins could be responsible for craniofacial *Dkk1* expression.

The impact of doubleridge CNEs on Dkk1 cis-regulation

In *doubleridge* mutant mice, *Dkk1* expression is dramatically reduced in the head, limbs and tail, resulting in fore limb polysyndactyly and kinked tail (Adamska et al., 2003; MacDonald et al., 2004). A further reduction of Dkk1 by combining *doubleridge* with *Dkk1*+/− alleles resulted in stronger phenotypes, such as anophthalmia, hydrocephaly and hypoplastic anterior nasal head structures. Given, that several *Dkk1* regulatory sequences are not located within the *doubleridge* location, or exhibit regulatory redundancy, we suppose that this might explain the relatively mild phenotype, in comparison to *Dkk1*−/− mice. However, we identified an optic cup/limb enhancer (CNE195), located within the *doubleridge* location. These data suggest that the loss of this particular sequence might be the reason for the *doubleridge* optic cup and limb phenotype. The polydactyly of *doubleridge* mutant embryos is caused by a broadened AER (Adamska et al., 2003). Vice versa, thalidomide mediated upregulation of *Dkk1* expression causes a reduction of AER formation during chicken development, leading to limb truncations (Knobloch et al., 2007; Knobloch et al., 2008). Therefore we suppose that the Dkk1 level has to be kept in a well defined threshold to control the AER mediated limb outgrowth. Furthermore, we found that two CNEs, CNE168 and CNE175 within the *doubleridge* locus promote reporter gene activity within a specific craniofacial domain, the mesenchyme of the vomeronasal organ. As *doubleridge*−/− embryos exhibit a loss of nasal craniofacial structures, the loss of these particular regulatory regions might be the reason for their craniofacial phenotype.

Combinatory and redundant regulatory properties of Dkk1 CNEs

We found that a well defined combination of CNEs was necessary to activate the reporter in several tissues. For example, the

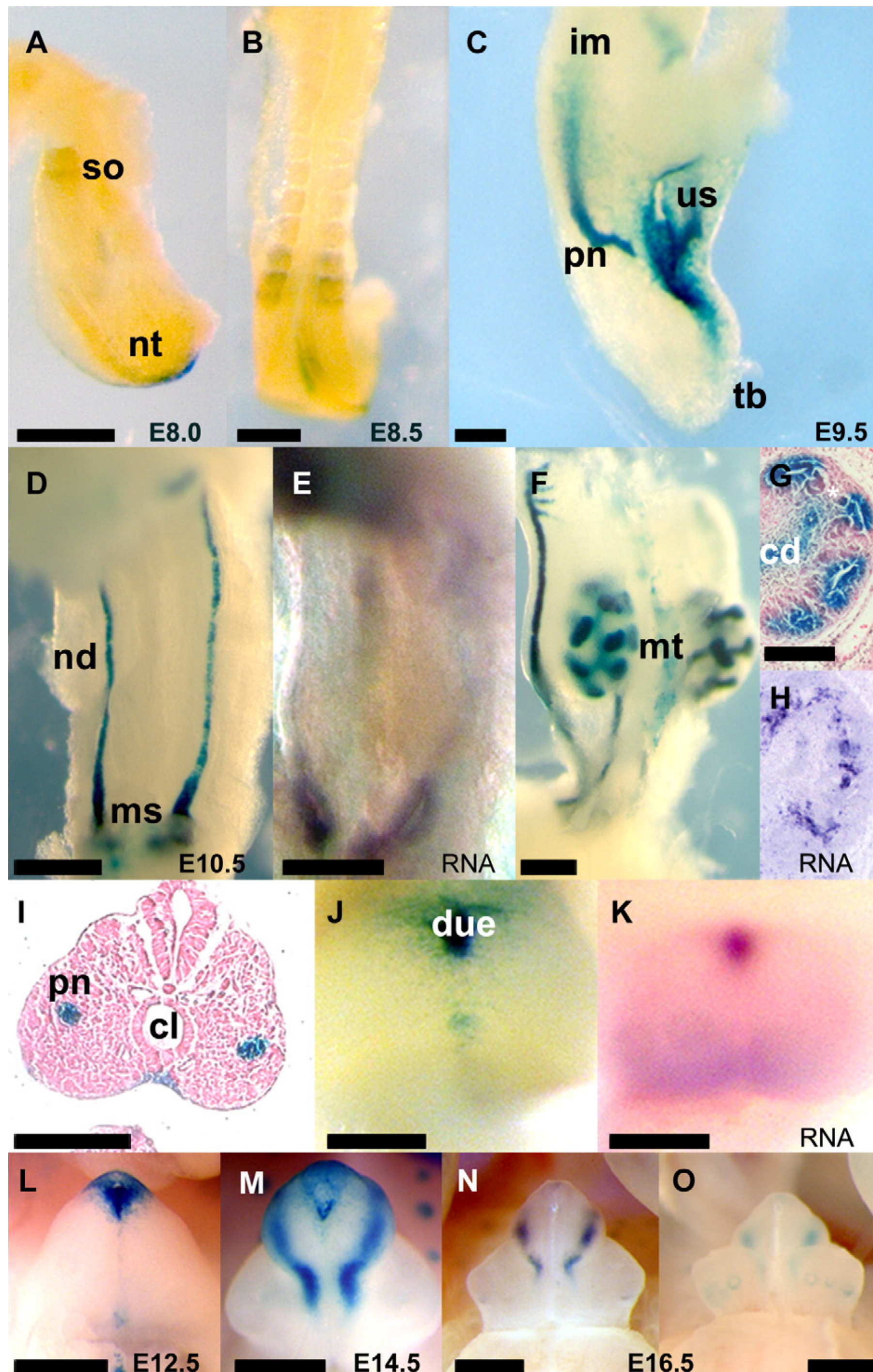


Fig. 8. Regulation of *Dkk1* during posterior embryonic development. (A–C, D, F, G, I, J, L–O) β-Gal staining is shown at different developmental stages, in comparison to the endogenous *Dkk1* expression pattern (E,H,K). (A, B) E8.0 and E8.5; gradient like reporter activity is evident in posterior most somites (so) and the posterior neural tube (nt). (C) Activity is detectable in intermediate mesoderm regions (im) that have aggregated posteriorly and built the pronephros (pn). *LacZ* expression is visible additionally in the urogenital sinus (us) and weakly in tailbud (tb). (D–H) Dynamics of *lacZ* and *Dkk1* expression during kidney development. (D and E) LacZ activity is evident in the nephric duct (nd) that posteriorly has built the mesonephros (ms), identical to *Dkk1* expression at E10.5. (F and G) *LacZ* expression is detectable in the urethric buds of the metanephros (mt), the collecting duct, and renal vesicles at E12.5, very similar to the endogenous *Dkk1* expression (H). (I–O) LacZ activity during external genitalia development. (I–K) Expression appears in the mesenchyme of the cloacal membrane at E9.5, the distal urethral epithelium (due) at E11.5 in comparison to *Dkk1* expression (K). (L–O) Reporter activity during later external genitalia development. (L) Activity intensifies in the due at E12.5. (M) At E14.5, Expression appears in the mesenchyme, surrounding the due. At E16.5, activity is dramatically decreased in male and female external genitalia (N and O). Scale bar: 100 µm in (C); 200 µm in (A,B,F–K); 500 µm in (D,E,L,M–O).

combination of at least CNE25, 114, 190 and 195 promotes expression in the eyelids (data not shown). Furthermore, our data revealed that a 5.7 kb genomic region, containing CNE190 and 195 can drive expression in the hypophysis and genital tubercle (Figs. 10A and B). However, these sequences alone are not able to mediate this expression. Thus, either a combination of these two CNEs is needed or the flanking sequences are required for this expression.

As revealed above, we found that all four CNEs tested individually mediated a similar level of reporter gene activation in lateral domains of the dorsal brain and the metanephros (shown for CNE25 in

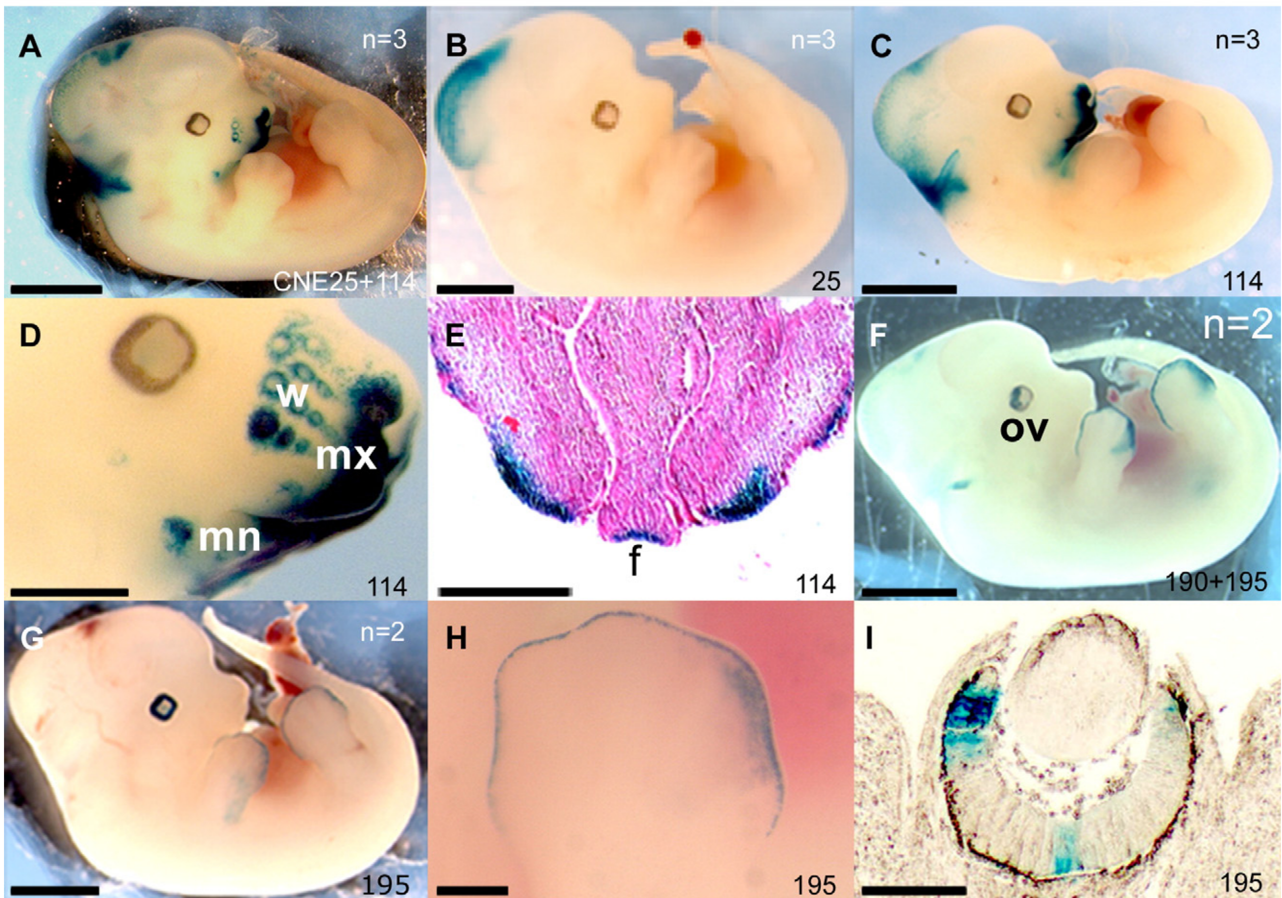


Fig. 9. The regulatory potential of *Tg107* CNEs. (A–D, F–H) Sideviews on β-Gal stained whole-mount embryos, and transverse sections is presented (E and I). (A) Reporter activity is evident in craniofacial domains promoted by CNEs 25 and 114. (B) CNE25 promotes reporter expression in the dorsal brain. (C and D) CNE114 alone mediates reporter activation in craniofacial expression domains. (E) CNE114 does not promote reporter expression in the vomeronasal domain. (F) A 5.7 kb sequence, containing CNEs 190 and 195 promotes expression in the optic cups and limbs. (G–I) CNE 195 alone mediates reporter expression in the limbs (H) and optic cups (I).

Figs. 10C and D). Thus, these *Dkk1* CNEs have redundant regulatory properties in these tissues. Regulatory redundancy has been previously shown for other gene regulatory sequences (Bagheri-Fam et al., 2006; Cretekos et al., 2008). However, sequence alignments of these CNEs did not reveal any homology (see above).

Identification of additional Dkk1 expression sites suggests novel functions of Dkk1 during development

We were able to extend the knowledge of the *Dkk1* expression pattern during mouse development. Like *Dkk1* expression in the pituitary of *Xenopus* embryos (Monaghan et al., 1999), we revealed that *Dkk1* expression in the 3rd ventricle extends into the posterior pituitary during mouse development, suggesting an evolutionary conservation and function of *Dkk1* during hypophysis development. *Dkk1* becomes downregulated after early hypophysis development and is absent at E14.5 (data not shown), indicating that Dkk1 might be involved directly during hypophysis development. As we found that a 5.7 kb genomic region, containing CNEs190 and 195 promotes reporter activity in the hypophysis, we suppose that activation of *Dkk1* expression is mediated by this regulatory region. As Wnt genes, associated signaling components and several antagonists exhibit

Table 1
The regulatory potential of *Dkk1* CNEs.

	n =	Mesencephalon	Metanephros	Hypophysis	Genital tubercle	Craniofacial[a]	Optic vesicle	Limbs[b]
CNE25;114;190;195	4/8	+	+	+	+	+	+	+
CNE25 + CNE114	3/6	+	+	–	–	+	–	–
CNE25	3/6	+	+	–	–	–	–	–
CNE114	3/4	+	+	–	–	+	–	–
CNE190 + CNE195	2/4	+	+	+	+	–	+	+
CNE190	4/11	+	+	–	–	–	–	–
CNE195	2/6	+	+	–	–	–	+	+

Summary of the regulatory potential of Dkk1 CNEs. Numbers of identical stained embryos out of total transgenic embryos are indicated. CNE 25, 114, 190 and 195 individually and in combination commonly activate the reporter in the mesencephalon and the metanephros. A 5.7 kb genomic region, containing CNEs 190 and 195 promotes activation in the hypophysis and the genital tubercle. CNE114 mediates craniofacial activation and CNE 195 promotes activation in the optic cups and limb buds.

[a] Mandibular, maxillar, tongue, hair follicle.

[b] AER, PNZ.

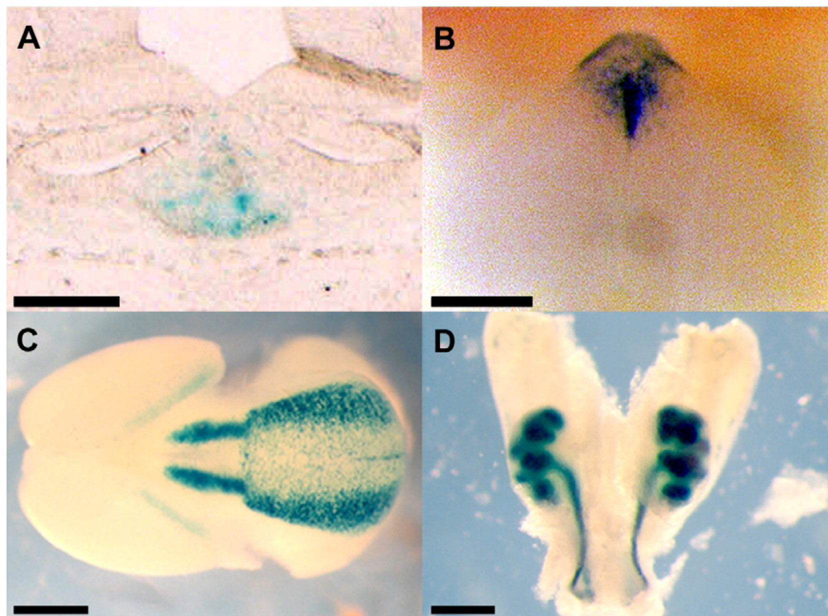


Fig. 10. Combinatory and redundant regulation of *Dkk1* expression. β-Gal staining of (A) transverse section or (B–D) whole mounts of different organs are shown. (A and B) A 5.7 kb genomic sequence, containing CNE190 + 195 promotes reporter expression in the pituitary and the external genital placode. CNE 25 tested in a single construct promotes reporter activitation in the dorsal brain (C) and the metanephros (D). Scale bar: 200 µm (A,B); 500 µm (D); 1 mm (C).

exclusive expression patterns and functions during hypophysis development, Dkk1 might directly function to inhibit Wnt signaling (Potok et al., 2008; Brinkmeier et al., 2007; Ai et al., 2007).

The dynamic expression patterns during kidney and external genitalia development suggest further functions of *Dkk1* during posterior embryonic development. We found that *Dkk1* is expressed already in the pronephros during mouse development like in *Xenopus* embryos (Monaghan et al., 1999), suggesting that early *Dkk1* expression and function is evolutionary conserved and not restricted to amphibians. Characteristic expression patterns and functions of several canonical Wnt family members exist during kidney development (e.g. Itaranta et al., 2002; Kuure et al., 2007; Lin et al., 2001). In these areas, intense canonical Wnt signaling drives branching nephrogenesis in fetal kidney explants (Iglesias et al., 2007), and exposure of kidney explants to Dkk1 causes an inhibition of canonical Wnt signaling, resulting in a reduction of uretic bud arborization (Iglesias et al., 2007). Furthermore, *Dkk1* is downregulated in *Lim1* mutant kidneys (Potter et al., 2007), suggesting that *Dkk1* is genetically located downstream of Lim1. This might be governed by direct regulation via Lim1 binding sites within the identified CNEs, as CNEs 25, 114, 190 and 195 contain conserved Lim1 binding sites (data not shown).

The transgenic mouse line revealed a dynamic *Dkk1* expression during external genitalia development. This expression ranges from the early urogenital sinus stage until E16.5, suggesting an involvement of *Dkk1* in genitalia patterning. Wnt genes also exhibit a characteristic expression and canonical Wnt signaling is required for external genitalia development (Gleason et al., 2006; Lin et al., 2008). Herein, β-catenin is required to maintain tissue integrity (Lin et al., 2008). Furthermore, it was recently shown that Wnt/β-Catenin signaling in the distal genital tubercle requires the activity of *Shh* during the outgrowth of the genital tubercle (Miyagawa et al., 2009) and that Wnt5a affects the development of the proximal cloacal plate (Nakata et al., 2009). These data reveal a well defined organization of Wnt signaling activity which might be controlled by the activity of *Dkk1*. Our first analyses of reporter activity during external genitalia outgrowth by analyzing combined *Dkk1*−/−; *lacZ* alleles already suggests a role of *Dkk1* in this context (data not shown). Thus, extension of the known *Dkk1* expression enables the discovery of so far unknown functions.

The regulation of Dkk1 transcription and the use for therapeutic approaches

In general, the modulation of Wnt signaling is of therapeutic relevance for tissue repair in different diseases (reviewed by Zhao et al., 2009). For example, multiple myeloma patients exhibit high levels of *Dkk1* in their blood serum (Tian et al., 2003). A treatment of these patients with autologous stem cell transplantation causes a decrease of Dkk1 in their serum and an elevation of bone formation markers (Politou et al., 2006). Furthermore, it was recently suggested for arthritis and multiple myeloma that affecting the Dkk1 mediated Wnt inhibition by the Dkk1 antagonist Rspo1 might be a useful therapeutic approach (Zhao et al., 2009). Several disease related mouse lines are used to monitor disease and cancer formation. Combining these mouse lines in combination with one *Tg107* allele, alterations in *Dkk1* expression would be easy to monitor via the reporter. Moreover, the knowledge about the regulatory potential of the identified CNEs enables the analysis of direct *Dkk1* targeting during these processes. Therefore, the *Tg107* mouse line might be useful as an in vivo model system for drug screening in order to establish therapeutic approaches for diseases based on a Dkk1/Wnt imbalance (reviews focusing on such diseases are given by Zhao et al., 2009; Niehrs, 2006).

Materials and methods

Generation of reporter constructs

For the generation of transgenic mice, genomic fragments were PCR amplified from BAC clones RP23-118N16, RP23-98J4, and RP23-182C8. The *Dkk1* promoter fragment, containing 470 bp upstream and 60 bp of the first *Dkk1* exon was PCR amplified with the oligonucleotides:

5′-AGGGGCGCGCCCAGCCCCACAGCAGAACACTCAAGCTCAC-3′ and 5′-CGCGGATCCGCCATCATTGTAAACACGGCCAAG-3′. This fragment was sub-cloned into the T-vector (Promega), and cloned into the *pPD46.21*

reporter vector with *SalI*/*BamHI* in frame to the *lacZ* reporter gene. The promoter was extended to a 2.785 kb promoter fragment by cloning via *HindIII*/*SphI*. The 1.8 kb promoter/reporter fragments were generated by *SwaI*/*NotI* restriction enzymes. CNEs were identified, using several data banks (http://ecrbrowser.dcode.org; http://ensembl.org; http://genome.lbl.gov/vista/index.shtml). Conserved sequences were PCR amplified with the following oligonucleotides (length of the cloned fragments is indicated in brackets): CNE25fw 5′-GCAACATGAAAGTAGAGCAAG-3′ CNE25bw, 5′-CACAGAAGTATGAATTGAAGC-3′ (1978 bp); CNE87fw 5′-CCAGTGCTTGGTCTCTGA-3′, CNE87bw 5′-CCTGTAGCTCCTATGAAAC-3′ (1066 bp); CNE114fw small 5′-GCTATGTTCTCACAGGGGAAA-3′, CNE114bw small 5′-TCAGCCCTGATAGTTTACA-3′ (641 bp); CNE114fwbig 5′-TTAACGTCACGTCTCCACAG-3′, CNE114bwbig 5′-TATAGCACCACCCTGTTCTC-3′ (2457 bp); CNE129fw 5′-CGATGTTATGTCTCTTGTCAC-3′, CNE129bw 5′-GGAGATGCAATCAACTCCTAC-3′ (361 bp); CNE140fw 5′-GATCAGATTGTCCACATGCAAC-3′, CNE140bw 5′-CATGTCTCCATTCTCCACCATC-3′ (1153 bp); CNE168fw 5′-GGTAGCATACTAGAAGAATCAG-3′, CNE168bw 5′-CCAACTAGGCCATCTTCTGAT-3′ (1725 bp); CNE175fw 5′-GCTGATTAGCAGGAGGTTC-3′, CNE175bw 5′-GGTGTTGGCTCACAACCATC-3′ (1008 bp); CNE190 + 195fw 5′-TAGTGAGAAGCTGGAAGCAG-3′, CNE190 + 195bw 5′-CCACTCTCAGTTCTATTGTGG-3′ (5743 bp); CNE190fw 5′-GAGGATGTGGAGTTTAAGGTC-3′, CNE190bw 5′-GTCCACTTTGCAGGAATGATC-3′ (456 bp); CNE195fw 5′-CTGAGCAACCAATTACTGTAC-3′, CNE195bw 5′-GCTTATTCTCTGGATTCCTAC-3′ (514 bp). Genomic fragments were individually sub-cloned in the T-vector (Promega), isolated with *NotI*/*Bsp120I* and cloned into the *NotI* linearized promoter vectors in combinations or alone.

Generation and genotyping of transgenic mice

For microinjection, *lacZ* reporter constructs were isolated from the vector backbones by cutting with *SwaI*/*NotI*. The excised insert fragments were separated by gelelectrophoresis and purified with the QIAquick PCR Purification Kit (Qiagen). 1.5 ng/µl DNA was microinjected into male pronuclei of fertilized *B6C3*/*F1* mouse oocytes, according to Gordon and Ruddle (1983). DNA of embryos and founder animals was isolated from Proteinase K-digested yolk sacs and tail biopsies. Genomic DNA was used as templates to identify transgenic mice with *lacZ*-specific primers 5′-GTTCCGTCATAGCGATAACGAG-3′ and 5′-CACTTACGCCAATGTCGTTATCC-3′. To establish the mouse line, transgenic animals were crossed with *C57BL*/*6* mice.

Whole-mount-β-galactosidase staining

Embryos were fixed and stained 4–20 h as previously described (Theil et al., 1999). LacZ stained embryos were postfixed in 0.4% PFA and paraffin embedded. 7–12 µm sections were counterstained with Eosin.

In situ hybridisation

Dkk1 in situ hybridisation of whole-mount embryos or on sections was performed as previously described (Grotewold et al., 1999).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2010.01.037.

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