Chapter 2

Missense Mutations in GDF-5 Signaling: Molecular Mechanisms Behind Skeletal Malformation

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1. Introduction

Members of the large transforming growth factor β (TGF-β) superfamily of secreted growth factors initiate cellular signal transduction via binding to and oligomerization of two different types of membrane bound serine/threonine kinase receptors termed type I and type II (Carcamo *et al.*, 1994, ten Dijke *et al.*, 1996, Massague, 2000). They execute important functions in early (e.g. gastrulation) as well as in later stages (e.g. patterning) of embryonal development, but are also essential for regulation of tissue homeostasis and repair in the adult organism (Rosen & Thies, 1992, Kingsley, 1994, Hogan, 1996, Reddi, 1998, Massague, 2000). A characteristic feature of this protein family is the high degree of promiscuity in the ligand-receptor interaction (for review see (Sebald *et al.*, 2004, Nickel *et al.*, 2009)). This is exemplified by the numeral discrepancy of a likewise large number of ligands - more than 30 ligands are known in mammals to date – and a comparably small number of receptors available for binding and signaling (Miyazawa *et al.*, 2002). Only 12 receptors exist in the TGF-β superfamily of which seven belong to the type I and five to the type II receptor subclass (Newfeld *et al.*, 1999). This implies that a given receptor typically binds more than one TGF-β member, but we usually see that even a particular TGF-β ligand binds more than one receptor of either subtype (for review see (Sebald *et al.*, 2004, Nickel *et al.*, 2009)). Noteworthy, another seemingly reduction in the signaling output is due to the fact that principally only two primary pathways are activated by all TGF-β members (Hoodless *et al.*, 1996, Nakao *et al.*, 1997). After ligand-dependent oligomerization of the single transmembrane receptors, the intracellular kinase domain of the type II receptor activates the type I receptor kinase domain by transphosphorylation of a type I receptor exclusive membrane-proximal glycine/serine-rich region, termed GS-box (Shi & Massague, 2003). This phosphorylation unleashes the binding site for a group of transcription factors called SMADs whose naming derives from their

homology to *Drosophila's* mothers against decapentaplegic (MAD) and the *C. elegans* protein Sma (Derynck *et al.*, 1996). Dependent on the nature of the type I receptor present in the TGF-β ligand-receptor signaling complex R-SMAD proteins (for receptor-regulated SMADs) either belonging to the so-called SMAD1/5/8 or the SMAD2/3 family become phosphorylated. Subsequently, the so activated SMAD1/5/8 or SMAD2/3 proteins form heteromeric SMAD complexes comprising one R-SMAD of either of the aforementioned subfamilies and the common mediator SMAD protein SMAD4. This heteromeric SMAD complex then translocates into the nucleus where it regulates gene transcription by functioning as a transcription or co-transcription factor (see Fig. 1) (Heldin *et al.*, 1997, Miyazono, 2000, Massague *et al.*, 2005).

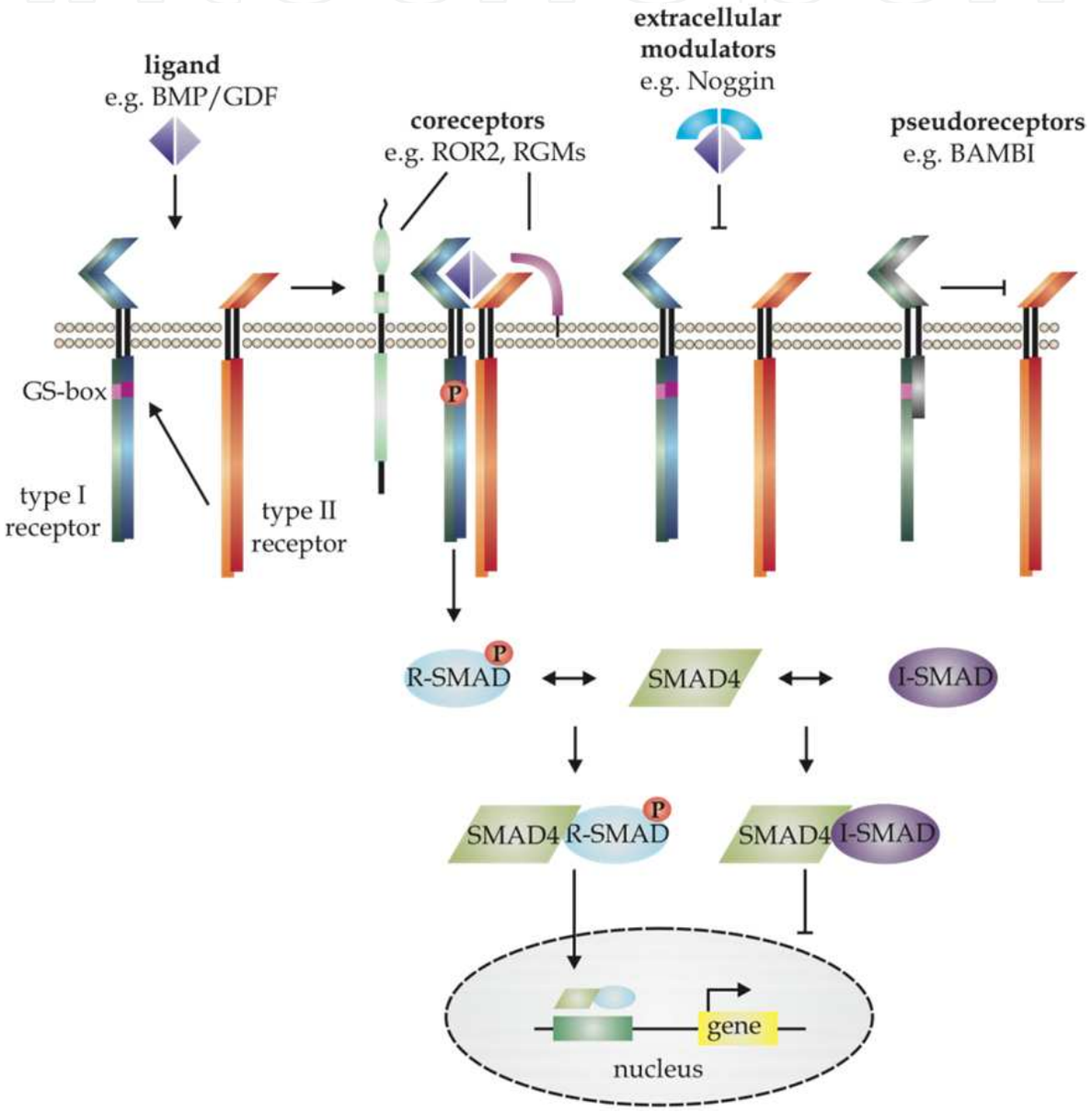

Figure 1. Signal transduction of BMPs and GDFs. Signal transduction is initiated by binding of the dimeric ligand to two types of transmembrane serine-/threonine kinase receptors termed type I and type II. Upon ligand binding the receptor chains oligomerize and the type II receptor transphosphorylates the type I receptor at the so-called GS-box thereby activating the kinase domain. Consequently, intracellular

downstream signaling components termed receptor-regulated SMADs (R-SMADs) are activated by phosphorylation. These R-SMADs then oligomerize with the common mediator SMAD (co-SMAD), SMAD4, translocate into the nucleus and in concert with other transcriptional modulators regulate target gene transcription. Regulation of this signaling pathway can occur at multiple levels as indicated. Thus, extracellular signaling modulators (e.g. Noggin, Follistatin) can bind to BMP/GDF ligands thereby preventing the interaction with their signaling receptors. On the membrane level coreceptors like ROR2 or members of the repulsive guidance molecule (RGM) family are thought to interact with the receptors and/or the ligands thereby amplifying the BMP/GDF signal. On the contrary, the pseudoreceptor BAMBI is an inhibitor of BMP as well as Activin signaling. The extracellular domain resembles the ligand binding interface of the type I receptors, while an intracellular kinase domain is lacking. The inhibitory function of the pseudoreceptor is potentially due to the formation of complexes with type I and/or type II receptors, thereby interfering with regular signal transduction. Amongst others, signal transduction can also be modulated intracellularly by the so-called inhibitory SMADs (I-SMADs), SMAD6 and SMAD7, where the I-SMADs compete with activated R-SMADs for interaction with SMAD4.

1.1. The multitude of biological functions of TGF-β members is established by a highly complex regulatory "cross-reactive" signaling network

Analysis of the patterning function of TGF-β members showed that they act as classical morphogens, i.e. the factors form a concentration gradient across the developing tissue and a specific cellular response is triggered dependent on the morphogen concentration (for review see (Wu & Hill, 2009)). A precise morphogenic function of an individual ligand can therefore only be explained in that either distinct tempero- and/or spatial distribution patterns of this ligand and its respective receptor(s) exist, which provide for specific signals at individual sites of action or in that the signaling event is tightly controlled by additional regulatory mechanisms. In the past years various studies identified a multitude of different components modulating the signal transduction of TGF-β members either outside the cell through secreted antagonists/modulator proteins (Ueno *et al.*, 1987, Smith & Harland, 1992, Francois *et al.*, 1994, Merino *et al.*, 1999b, Shimmi & O'Connor, 2003), at the cell surface level via activating coreceptors or deactivating pseudoreceptors or extracellular matrix components (Lopez-Casillas *et al.*, 1993, Onichtchouk *et al.*, 1999, Gray *et al.*, 2002, Wiater & Vale, 2003, Babitt *et al.*, 2005, Samad *et al.*, 2005, Lin *et al.*, 2007), or in the cell interior through proteins interacting with the receptors, SMAD components or via influencing receptor turnover or degradation (see Fig. 1) (Zhu *et al.*, 1999, Wotton & Massague, 2001, Chen *et al.*, 2006). The majority of these modulating mechanisms again involve proteins, which themselves exhibit promiscuous binding to several partners, thus resulting in a highly complex regulatory "cross-reactive" network. It thus seems logical that attempts or incidents, which *in vitro* seem to manipulate individual interactions by a defined mechanism, will *in vivo* inevitably lead to a massive intervention in an interweaved signaling network with established equilibrium of cross-interacting partners.

1.2. What can be learned from individual gene deletions?

Due to the morphogen's inherent coupling of ligand concentration and signaling activity it is therefore expected, that mutations causing an alteration in signaling capacities become

visible in a broad variety of different phenotypes. Consistently, a vast number of mutations could be correlated with inherited diseases (see OMIM database). Although often a clear correlation between mutation and phenotype can be drawn, in most of the cases the molecular mechanism translating the individual mutation into the corresponding phenotype remains unclear. An alternative strategy to identify functions of individual signaling components in the above-described signaling network is to eliminate their signaling input or function by null mutations. In the past decades a large number of knockout mice have been generated (TGF-β ligands, receptors, modulator proteins, etc.) and the loss of individual or combinations of genes of the TGF-β signaling network were analyzed in detail in hetero- as well as in homozygous situations (Zhao, 2003). Surprisingly, given the importance of TGF-β members for embryonic development and organogenesis, deletion of some genes of this superfamily did not result in prominent phenotypes (e.g. BMP-6) indicating that others can maximally compensate for a loss of these signaling components. On the other extreme some individual gene deletion resulted in embryonic lethality (e.g. BMP-2 or BMPR-IA) indicating that these components might occupy invariable key signaling positions, but thereby also impeding a detailed elucidation of gene function during development. In these situations, gene function was often further analyzed using conditional knockout mice to overcome lethality or to allow a cell- or tissue-specific deletion of the target gene to study the gene function in a more restricted environment. For some of the genes investigated it could be demonstrated, that a multitude of biological functions are strongly connected to the presence of one gene product in a strict temporal and spatial manner. For instance, it could be demonstrated for the receptor BMPR-IA that this receptor is essential for the formation of mesoderm during embryogenesis, (Mishina *et al.*, 1995) but also for the differentiation and proliferation in postnatal hair follicles (Andl *et al.*, 2004). However, these examples should emphasize the main problem of identifying individual relations between the factors and their biological function in such regulatory signaling networks. For the analysis of such mutation/function relations it is essential that a particular mutation translates into a visible phenotype and that this mutation does not result in embryonic lethality.

2. The role of GDFs in limb development

Astonishingly, within the complex machinery of TGF-β signaling only a few components seem to fulfill these criteria and for those a collection of mutations have been identified in the past years. One of these genes encodes for growth and differentiation factor 5 (GDF-5), which – like the other members of the TGF-β superfamily – binds as secreted signaling molecule to a defined subset of type I and type II receptors and initiates the activation of downstream signaling cascades. The biological role of GDF-5 *in vivo* became first apparent from the genetic analysis of the *brachypodism* mice (*bp*) (Storm *et al.*, 1994), which also finally led to the discovery of GDF-5, -6 and -7. In *brachypodism* mice length and number of bones in the limbs are altered, but the axial skeleton does not seem to be affected (Gruneberg & Lee, 1973). It has already been suggested in the early 1980's that the *bp* mutation very likely disrupts a signaling event, which naturally leads to mesenchyme aggregation and chondrogenesis in the limb (Owens & Solursh, 1982). Initially three independent *bp*

mutations have been described, which were all mapped to the *GDF5* locus on chromosome 2 all resulting in a frame-shift of the open reading frame and thus basically representing *GDF5* null mutations (Storm *et al.*, 1994). As a result of the *bp* mutations several long bones show reduced length and the first two phalanges in the digits II-V are replaced by a single bony element in all four extremities (Gruneberg & Lee, 1973). It is important to note that despite *GDF5* mRNA expression was reported to occur in a variety of non-skeletal tissues, e.g. the uterus, placenta, brain, heart, lung, kidney, etc., *bp* mice are fertile and do neither show behavioral abnormalities nor do they exhibit any morphological changes outside a few defined limb elements.

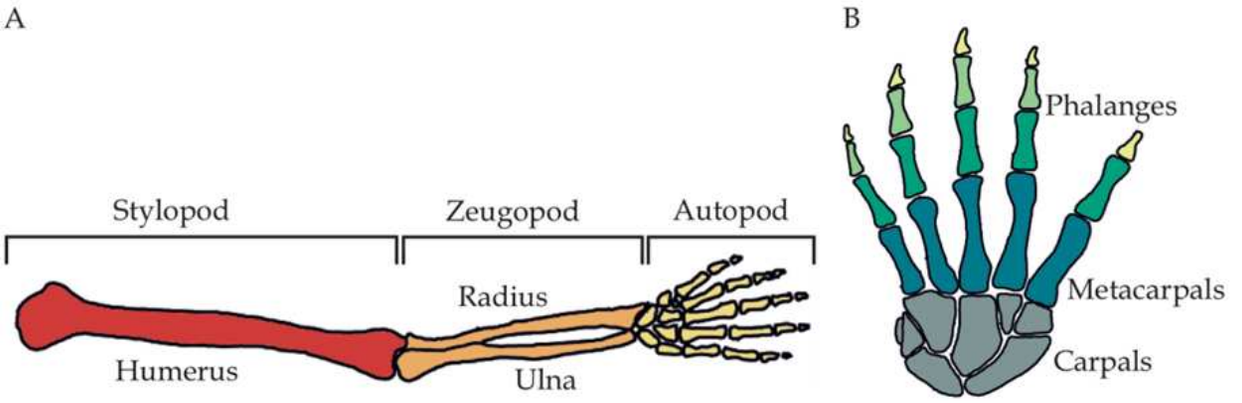

Figure 2. Schematic representation of the skeletal elements of a human limb and autopod.
A) Skeletal elements of a human limb. The stylopod gives rise to the humerus, the most proximal element of the limb skeleton, followed by the bony elements of radius and ulna, which derive from the zeugopod. Most distally, the autopod forms the bones of the hand.
B) Representation of the bony elements of the human autopod subdivided into the bones of the wrist (carpals), palm (metacarpals) and digits (phalanges).

The elements of the vertebrate limb originate from mesenchymal cells that first condense and subsequently initiate a differentiation program leading to the production of cartilage and bones in a highly defined fashion. These skeletal elements develop from single condensations in a proximal-to-distal sequence, which first grow and then branch and segment starting with the condensation forming the humerus at 10.5 days post coitus (dpc) (Wanek *et al.*, 1989, Storm & Kingsley, 1996, Francis-West *et al.*, 1999). The humerus aggregate then branches distally at 11.5 dpc thereby forming the condensations for the radius and the ulna (for nomenclature see Fig. 2). The digits develop as continuous structures called digital rays, which lengthen distally during further outgrowth. In order to build regular hands or feet the rays will then (13.5 - 15.5 dpc) be further separated in a sequential segmentation process to form the metacarpals and the phalanges. In mice *GDF5* mRNA is first detectable in the developing forelimb at 11.5 dpc in the proximal and distal region that will later form the shoulder and the elbow (Storm & Kingsley, 1996, Francis-West *et al.*, 1999). At 12.5 dpc GDF-5 is additionally expressed within the developing digital ray at a site that likely forms the future joint between the metacarpals and proximal phalanges. One day later at 13.5 dpc *GDF5* mRNA is expressed in the developing rows of carpals and in an additional stripe across the digital rays, with the sites coinciding with

developing joints in the wrist and the first interphalangeal joint (Storm & Kingsley, 1996). At 14.5 dpc the segmentation process seems completed, an additional stripe of GDF-5 expression separates the developing intermediate and distal phalanges and now all elements of a mice forelimb are defined and undergo chondrogenesis (Fig. 3) (Storm & Kingsley, 1996).

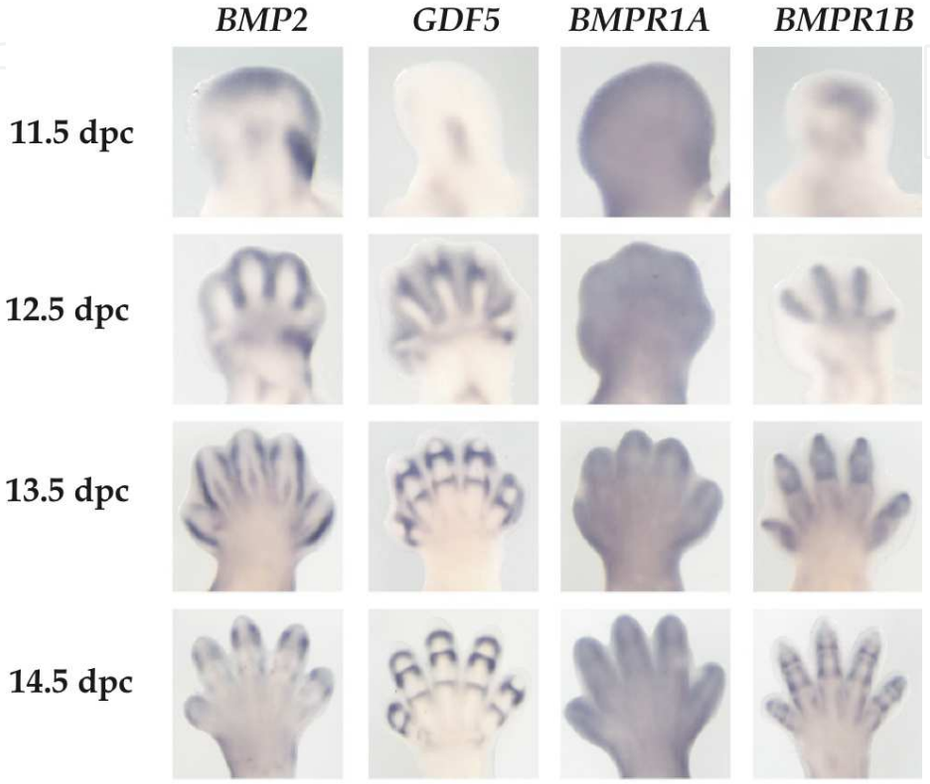

Figure 3. Expression pattern of *BMP2*, *GDF5*, *BMPR1A* and *BMPR1B* in the developing mouse fore limb.

Whole-mount in situ hybridization of *BMP2*, *GDF5* and their receptors *BMPR1A* and *BMPR1B* in a mouse fore limb at different embryonic stages. *GDF5* expression marks the developing cellular condensations. At 11.5 dpc *GDF5* is expressed in regions later forming shoulder and elbow. At 12.5 dpc *GDF5* is additionally visible in the future joints between the metacarpals and proximal phalanges. Later it is expressed in a stripe of the digital ray corresponding to the future interphalangeal joints separating the proximal from the intermediate (13.5 dpc) and the intermediate from the distal phalanges (14.5 dpc). *BMP2* expression is seen in the apical ectodermal ridge, the underlying mesenchyme and at the posterior side of the limb at 11.5 dpc. One day later, *BMP2* expression is mainly restricted to the interdigital mesenchyme as well as to the posterior wrist forming region, the wrist and the distal joints of radius and ulna. At 13.5 dpc *BMP2* expression can be localized to a region surrounding the cartilage condensations of the dorsal tendons, whereas at 14.5 dpc it is mainly found around the regions of future interphalangeal joints. *BMPR1A* shows a more or less uniform expression throughout the whole developing mouse limb at all stages depicted above. In contrast, *BMPR1B* expression at 11.5 dpc is restricted to developing condensations of the digit anlagen. Later, at 13.5 dpc 14.5 dpc, *BMPR1B* expression can be found in regions of the future interphalangeal joints.

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The full process of joint formation occurs in three steps: First, special regions with high cell densities so-called interzones are formed corresponding to the stripes across the developing cartilage elements. Second, apoptosis leads to the removal of cells in the center of this interzone. Together with changes in the extracellular matrix on neighboring cells this creates a three-layered structure characteristic for the developing joint. Third, at both extremes of the interzone differentiation of the articular cartilage takes place leading to a fluid-filled gap between the (now segmented) skeletal elements (Haines, 1947, Mitrovic, 1978, Craig *et al.*, 1987). The above observations highlight GDF-5 as one of the earliest markers for joint formation, whose mRNA can be detected in the developing joint 24 to 36h prior to visible morphological changes in the interzone and its expression continues for 2 to 3 days (for details see Fig. 4). The reduction of the number of phalanges in the *brachypodism* mouse, which is basically a *GDF5* knockout mouse, is likely due to a failure in the segmentation in the digital rays (Storm *et al.*, 1994). In *bp* mice limb-bud development as well as the condensations for the initial digital rays seem normal, but during segmentation of the digital rays during 12.5 to 14.5 dpc the formation of an interzone leading to the separation of proximal and intermediate phalanges is absent in *bp* mice. However, as GDF-5 is expressed in all synovial joints in wildtype mice and not just in the first interphalangeal joints of digits II to V it seems apparent that GDF-5 cannot be the sole factor for the formation of all joints in the whole limb (Storm & Kingsley, 1996). Without knowing the nature and molecular functions of GDF-5 Hinchliffe and Johnson in 1980 already suggested that the *brachypodism* phenotype might be caused by the disruption of a **pattern** (of various factors) that determines the location of joints in the limb (Hinchliffe & Johnson, 1980). As GDF-5 shares between 80 and 86% amino acid sequence identity in its C-terminal mature part with GDF-6 and GDF-7 and the latter factors are also expressed during limb development it seemed logical to assume that these factors might compensate for the loss of *GDF5* in the *brachypodism* mutations (Storm & Kingsley, 1996). This hypothesis whether the two GDF-5 family members GDF-6 and GDF-7 can either substitute in case of a loss of *GDF5* or act in a synergistic manner was again tested by generating knockout animal models.

Both genes *GDF6* and *GDF7* are expressed in and around the developing joint (Hattersley *et al.*, 1995, Wolfman *et al.*, 1995), furthermore the mRNA expression pattern does not strictly overlap with that of *GDF5* (Wolfman *et al.*, 1997). Strong mRNA levels of *GDF6* can be observed in elbow and the carpal joints as well as the perimeter of the digital ray, whereas *GDF7* expression is restricted to the proximal interphalangeal joint (Settle *et al.*, 2003). Indeed, studies on *GDF6* knockout mice show fusions in joints different from those seen in the *brachypodism* mice - in *GDF6*$^{-/-}$ mice fusions of specific bones in the wrist and ankle correlate with the strongest *GDF6* expression in wildtype mice - possibly suggesting that a particular member of the GDF-5/6/7 family might be responsible for the formation of a subset of joints in the limb system (Settle *et al.*, 2003). Expression analysis using other joint markers such as *GDF5* (Storm & Kingsley, 1996), *PTHRP* (Parathyroid hormone-related protein, (Lanske *et al.*, 1996, Vortkamp *et al.*, 1996)) or *DELTAEF1* (a zinc-finger homeobox transcription factor, (Takagi *et al.*, 1998)) shows that the earliest stages of joint formation also occur in the absence of *GDF6* expression, but similar to the *brachypodism* mutations these morphological changes do not proceed and thus segmentation of these skeletal elements is

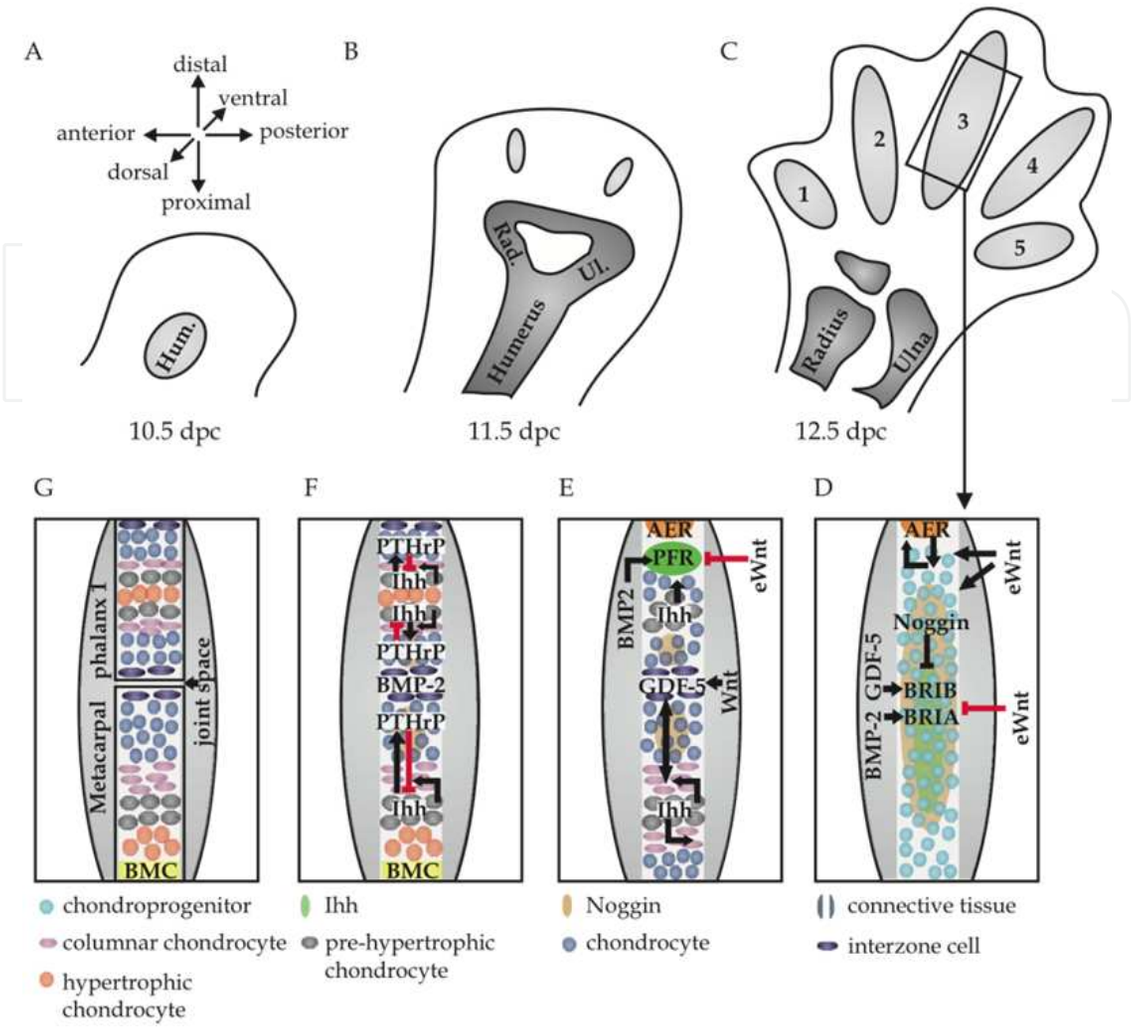

Figure 4. Schematic representation of limb bud outgrowth and determination of digit identities. A-C) Limb bud outgrowth. During limb bud initiation morphogen gradients determine the three main axes of the limb: proximo-distal, antero-posterior and dorso-ventral. Development of these gradients is under control of specific signaling centers such as the apical ectodermal ridge (AER) providing a proximo-distal gradient, the zone of polarizing activity (ZPA) producing an anterior-posterior gradient and the dorsal and ventral ectoderm establishing a dorso-ventral signal, thereby generating a morphogenic field inheriting the information for skeletal pattern formation (for review see Tickle, 2003 & 2006; Zeller, 2009). Skeletal elements of the vertebrate limb originate from mesenchymal cells that condense to form the cartilage anlagen, which develop in a proximo-to-distal manner starting with the condensation forming the humerus at 10.5 dpc. The humerus aggregate then branches distally at 11.5 dpc thereby forming the condensations of radius and ulna. The digits develop as continuous structures termed digital rays, which lengthen distally during further outgrowth. In order to build regular hands the rays will then (13.5 - 15.5 dpc) be further separated in a sequential segmentation process to form the metacarpals and the phalanges. D) Formation of the initial condensation in the human autopod. Distal mesenchymal cells under control of fibroblast growth factors (FGFs) derived from the AER and ectodermal Wnts (eWnts) remain in an undifferentiated, proliferative state. As cells escape from AER signaling they start to differentiate into prechondrogenic cells and later into chondrocytes, whereas chondrogenesis is negatively regulated by eWnt/β-catenin signaling. Mesodermally derived BMPs as well as GDF-5 positively influence differentiation by signaling via type I receptors BMPR-IA and BMPR-IB expressed in the chondrogenic precursor cells. E) Elongation and segmentation of the digit

condensations. Directed outgrowth of the condensations is achieved by BMP signaling in a region termed phalanx-forming region (PFR). This process is negatively regulated by eWnt signaling. Within the condensation pre-hypertrophic chondrocytes arise expressing Ihh, which positively influences PFR located BMP signaling. At the side of the future joint locally acting Wnt signals derived from the surrounding mesenchyme induce the differentiation of chondroprogenitor cells into flatened interzone cells expressing GDF-5. This process is encouraged by Ihh signaling from pre-hypertrophic condrocytes. Furthermore, GDF-5 and Ihh positively influence proliferation of columnar chondrocytes. F-G) Cavitation of the joint and growth of the digit. Ihh induces parathyroid hormone-related peptide (PTHrP) expressed in proliferative columnar chondrocytes underneath the future joint. PTHrP itself is a negative regulator of Ihh expression, thereby forming a negative feedback loop with Ihh. Interzone cells express BMP-2, which has a role in regulating apoptosis of these cells, thereby forming the joint cavity. The establishment of the so-called growth plate initiates further growth of the digit. This region is composed of zones of progressively differentiated chondrocytes: proliferating, columnar chondrocytes, followed by pre-hypertrophic chondrocytes expressing Ihh and finally hypertrophic chondrocytes eventually undergoing apoptosis thereby giving rise to the formation of the bone marrow cavity (BMC).

halted (Settle *et al.*, 2003). In contrast to *GDF5*$^{-/-}$ mice, which had fusions restricted to synovial joint, *GDF6*$^{-/-}$ mutants also showed defects in the cartilage and ligament structures of the middle ear and the coronal suture (a non-synovial joint) in the skull (Settle *et al.*, 2003). Analysis of the *GDF5*/*GDF6* double knockout mouse showed additional skeletal defects with many bones being strongly reduced in length or even being absent. As these defects are not observed in either one of the single knockout mice and are also observed in synovial joints outside the limbs it suggests that GDF-5 and GDF-6 act synergistically during the formation of specific joints (Settle *et al.*, 2003).

For GDF-7 function the effects in *GDF7*$^{-/-}$ mice are subtler and no changes in the skeletal patterning have been observed (Settle *et al.*, 2001). The phenotypes described comprise abnormal vesicle development in male mice (Settle *et al.*, 2001), smaller cross-sectional diameter of various long bones (Maloul *et al.*, 2006) and minor differences in tendon and ligament structures (Mikic *et al.*, 2006). A possible explanation for the very mild phenotype seen in *GDF7*$^{-/-}$ mice might be due to the upregulation of *GDF5* and *GDF6* mRNA expression above levels seen in wildtype mice leading to a partial compensation in the absence of *GDF7* (Mikic *et al.*, 2006). The above-described effects seen upon single or double deletion of GDF members indeed underline that GDF-5 alone, despite its patterning structure throughout the skeleton, does not induce the joint forming process in all joints of the developing limb. Moreover, it rather acts only on specific joints or might address additional ones throughout the limb in combination with GDF-6 or other factors (possibly in varying ratios) giving rise to the hypothesis that additional morphogens, e.g. members of the BMP superfamily, contribute to joint formation *in vivo*.

This idea that GDF-5 possibly acts via a defined combination with other factors to induce and maintain joint formation is supported by overexpression studies applying either locally ectopically GDF-5 protein (Storm & Kingsley, 1999) or by expressing GDF-5 systemically via retroviral transfection (Francis-West *et al.*, 1999). Interestingly, implantation of agarose beads soaked with recombinant GDF-5 into the limbs of chicken embryos did not lead to the development of additional ectopic joints. Instead, GDF-5 stimulated cartilage growth of

existing cartilage, which - dependent on the location of the implantation - could even interfere with joint development (Storm & Kingsley, 1999). Studies using developing limbs of mice show similar results, implanting recombinant GDF-5 in hind limbs at 12.5 or 13.5 dpc showed that GDF-5 stimulated growth of currently present cartilage cells whereas the interdigital mesenchyme did not respond to GDF-5 treatment after 12.5 dpc. This different response of both cell types could also be seen when different cartilage differentiation markers such as Collagen2 and Indian hedgehog *(IHH)* were analyzed with both markers being induced upon GDF-5 treatment in the existing cartilage but not in the interdigital mesenchymal cells (Storm & Kingsley, 1999). This suggests that the different cells present in the developing joints lose their GDF-5 responsiveness at different times. GDF-5 can thus be considered as a pro-chondrogenic factor that acts in a stage-dependent manner and is required but not sufficient for joint formation.

3. Disorders in limb development

A group of skeletal malformation diseases observed in humans, i.e. brachydactyly, symphalangism and chondrodysplasia, exhibits similar limb deforming phenotypes as observed in *brachypodism* mice suggesting that similar mechanisms and factors are affected in humans (for review see (Temtamy & Aglan, 2008, Mundlos, 2009)). All phenotypes describe skeletal malformations of extremities – especially of the phalanges – caused by abnormalities in cartilage development. Typically all the brachydactyly-causing mutations affect the formation of synovial joints due to a deregulation of chondrocyte proliferation and/or differentiation. The classification of the different diseases has initially been done by examining the skeletal malformation phenotype (Bell, 1951). Genetic analyses later revealed disease-causing mutations not only in GDF-*5*, but also in other TGF-β ligands, receptors or modulator proteins as well as in other differentiation factors. Nowadays the different brachydactyly phenotypes are classified into eight different forms (BDA1-3, BDB1-2, BDC, BDD, BDE), which show clear differences regarding affected phalanges (see Fig. 5).

Of those the brachydactylies BDA1, BDD and BDE are caused by genes that are seemingly unrelated to the TGF-β/BMP signaling pathway. In BDA1, which is characterized by shortened intermediate digits in all phalanges, inactivating mutations in the gene encoding for the secreted morphogen of the Hedgehog family Indian hedgehog (*IHH)* seem to be the molecular cause (Gao *et al.*, 2001, Liu *et al.*, 2006). Indian hedgehog is regulating chondrocyte proliferation and is also required for ossification of endochondral bones (St-Jacques *et al.*, 1999, Karp *et al.*, 2000). The skeletal malformation phenotype resembles that of the *IHH*$^{-/-}$ knockout mice (St-Jacques *et al.*, 1999) and suggested that binding to the receptor Patched (PTCH) and its subsequent activation is impaired in patients suffering from BDA1. Modelling of a potential receptor interaction of IHH on the basis of the crystal structure of Sonic hedgehog bound to the hedgehog antagonist HHIP indicates that the four missense mutations at position Gly95, Asp100, Glu131 and Thr154 inactivate IHH via two different mechanisms (Bosanac *et al.*, 2009). The mutations of Gly95, Asp100 or Glu131 disrupt the conserved calcium coordination site present in hedgehog proteins, which was shown to be

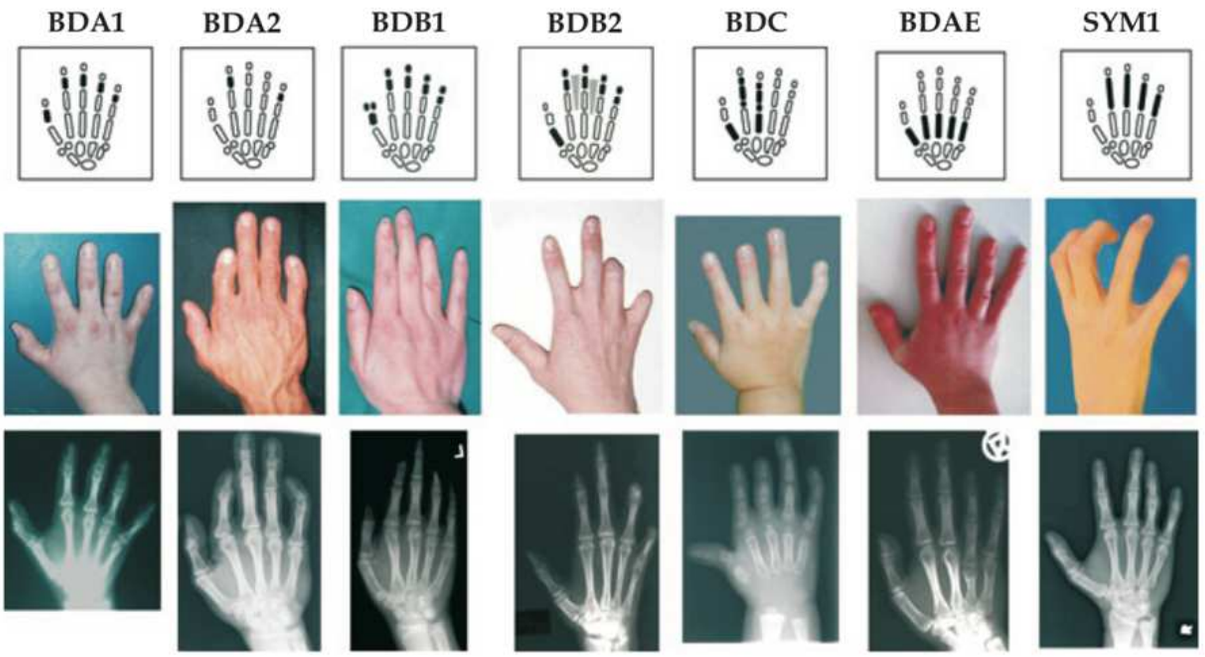

Figure 5. Clinical features of non-syndromic brachydactylies. In the top row, schematic representations of human hands depict specific phalanges and interdigital tissue affected in each skeletal malformation disease. Typical clinical features of hands are shown in the middle, corresponding X-rays underneath. Reprinted from Clinical Genetics (2009) 76, 123-136, S. Mundlos, "The brachydactylies: a molecular disease family", Copyright 2011, with permission from John Wiley and Sons.

required for high-affinity receptor binding (McLellan *et al.*, 2006, Gao *et al.*, 2009, Guo *et al.*, 2010). For the fourth mutation - T154I - identified recently no clear mechanistic explanation can be given, however based on the IHH 3D model Thr154 is located in close proximity to the other BDA1-associated missense mutations (Liu *et al.*, 2006) and thus possibly also interferes with receptor binding. Although neither IHH nor its receptors directly bind to TGF-β signaling components, BMP and IHH signals interact at various stages to regulate chondrocyte development. First of all, it has been shown that treatment of limb explants with the BMP antagonist Noggin leads to a decreased expression of *IHH* message (Minina *et al.*, 2001). Later Seki and Hata found that the *IHH* gene is a direct target of the BMP/SMAD signaling pathway due to the fact that GC-rich boxes in the promoter region of *IHH* confer binding of SMAD4 (Seki & Hata, 2004). This allows an upregulation of *IHH* expression in response to BMP signals. In the GDF-5 implantation experiments performed by Storm and Kingsley the GDF-5 dependent increase in the *IHH* mRNA message was used as a marker for chondrocyte differentiation (Storm & Kingsley, 1999). Secondly, there also seems to be a positive feedback loop as in chicken ectopic expression of IHH leads to an increased expression of BMP-2 and BMP-4 and similar results could be obtained in mice using transgenic animals in which the *IHH* gene expression is driven by a *COL2* promoter (Pathi *et al.*, 1999, Minina *et al.*, 2001). However, the effects of the deactivating IHH mutations in BDA1 are not exclusively transmitted via its direct regulatory roles on the BMP signaling pathway, besides the above described feedback loop between IHH and BMP pathways, both factors also exhibit independent functions in chondrocyte development (Minina *et al.*, 2001).

The brachydactylies BDD and BDE are characterized by a shortened distal phalanx in finger I and shortened metacarpals in fingers I to V, respectively. In both diseases mutations in the *HOXD13* gene seem to be the molecular cause (Caronia *et al.*, 2003, Johnson *et al.*, 2003). HOXD proteins represent homeobox transcription factors and disruption of the 5′ *HOXD* genes *HOXD11*, *HOXD12*, and *HOXD13* in mice have shown that these transcription factors exhibit important position-specific functions during limb development (Davis & Capecchi, 1996, Villavicencio-Lorini *et al.*, 2010). Two of three mutations described, I314L and Q371R seem to disrupt binding of the HOXD transcription factor to its target DNA site as deduced from structural modeling of the protein:DNA complex (Johnson *et al.*, 2003, Zhao *et al.*, 2007). Although the amino acid replacement is rather conservative, the leucine sidechain seems to introduce a steric hindrance to a neighboring pyrimidine base of the bound target DNA possibly altering the specificity for DNAs containing either a thymine or a cytosine in this sequence. For the second mutation, serine 308 to cysteine, it is difficult to deduce a molecular mechanism explaining the skeletal phenotype. Serine 308 located in the homeobox domain of HOXD13 is not in contact with the DNA and placed in a less conserved region, thus misfolding of the HOXD13 protein due to the different sidechain size and polarity of the introduced cysteine residue might explain the altered HOXD13 function. The effect of both mutations on DNA binding was however confirmed experimentally by electrophoretic mobility shift assays (EMSA) (Johnson *et al.*, 2003). Similar to BDA1 a direct regulatory or physical interaction of HOXD proteins and members of the TGF-β/BMP pathway is not apparent and thus it seems unclear at first sight whether the skeletal malformation phenotype of the HOXD13 mutants results from an independent parallel disturbed signaling pathway involved in limb development or whether HOXD13 might be an upstream or downstream target of the TGF-β/BMP signaling cascade. Suzuki *et al.* have found that both HOXA13 and HOXD13 transcription factors can enhance transcription of the *BMP4* promoter and may thus increase BMP expression (Suzuki *et al.*, 2003). Recently the group of Stefan Mundlos investigated the effect of the *HOXD11*, *-12*, *-13* and *HOXA13* genes on joint formation in mice and discovered that HOXD13 can directly bind and regulate the *RUNX2* promoter, whose activation is crucial for formation of cortical bone (Villavicencio-Lorini *et al.*, 2010). Studies using mice with defective *HOXA13* revealed that upon loss of *HOXA13* function mRNA expression for *GDF5* is downregulated, whereas mRNA for *BMP2* is upregulated (Perez *et al.*, 2010). As HOXA and HOXD proteins might form regulatory complexes, BDE initiating mutations in *HOXD13* may thus act via altering a defined concentration balance between GDF-5 and BMP-2 in the developing joint.

3.1. Disrupted GDF-5 signaling correlates with impaired joint formation

The other brachydactyly forms are caused by mutations in either *GDF5*, or other *BMP* genes, BMP receptors or modulator proteins thereby highlighting the central regulatory role of the GDF/BMP signals for proper joint formation. Mutations in the *GDF5* gene are found in brachydactylies of the type BDA1, BDA2 and BDC, but also in symphalangism and multiple synostosis syndrome phenotypes as well as in chondrodysplasias of the Grebe, Hunter-Thompson and DuPan type, which are more severe skeletal malformation diseases possibly

due to the fact that in the latter syndromes the mutations in *GDF5* are homozygous or compound heterozygous (see Table 1). Mutations in the BMP type I receptor BMPR-IB as well as a duplication of an about 6kb element in the 3′ regulatory untranslated domain of the *BMP2* gene also lead to brachydactyly of the type BDA2 (Lehmann *et al.*, 2003, Lehmann *et al.*, 2006, Dathe *et al.*, 2009). Mutations in the orphan tyrosine receptor kinase ROR2, which might possibly act as a GDF-5 specific coreceptor thereby influencing receptor activation of this TGF-β member, lead to brachydactyly of the type BDB1 (Oldridge *et al.*, 2000, Schwabe *et al.*, 2000). Amino acid exchanges in the BMP modulator protein Noggin are observed in patients suffering from brachydactyly type B2 (BDB2) (Lehmann *et al.*, 2007). As there is a wealth of structural and functional data available for almost all of the above-mentioned factors a more in-depth analysis can be performed to analyze the molecular mechanism behind these disease-causing mutations.

3.2. Mutations interfering with BMPR-IB kinase activity and signaling

So far three mutations in the BMP type I receptor BMPR-IB could be correlated with brachydactyly BDA2. In the BMP/GDF signaling pathway three type I receptors, BMPR-IA (Alk3), BMPR-IB (Alk6) and ActR-I (Alk2) can be addressed by the different ligands for binding and signaling (Sebald *et al.*, 2004). *In vitro* interaction analyses show that GDF-5 can bind only to BMPR-IA and BMPR-IB with affinities in the nano-molar range (Nickel *et al.*, 2005), whereas it shows no measureable interaction with the type I receptor ActR-I (Heinecke *et al.*, 2009). These and other *in vitro* studies also showed that GDF-5 interacts preferentially with BMPR-IB exhibiting a 10 to 15-fold higher affinity for BMPR-IB than for BMPR-IA (Nickel *et al.*, 2005, Heinecke *et al.*, 2009). Furthermore, performing a more *in vivo*-like radioligand binding assay in order to analyze the interaction of radiolabeled GDF-5 via chemical crosslinking to cells that were either transfected with the different type I and type II receptors or endogenously express BMP receptors, an exclusive binding of GDF-5 to BMPR-IB could be detected (Nishitoh *et al.*, 1996). Despite this rather strong binding specificity of GDF-5 to BMPR-IB on whole cells measuring transcriptional activation in mink lung cells transfected with different combinations of BMP type I and type II receptors showed that GDF-5 can activate SMAD signaling via BMPR-IB **and** BMPR-IA with almost identical efficiency (Nishitoh *et al.*, 1996). However, BMPR-IA cannot substitute for BMPR-IB in all GDF-5 initiated signals, e.g. induction of the osteogenic marker alkaline phosphatase (ALP) by GDF-5 is observed in the murine pro-chondrogenic cell line ATDC5, which does not express BMPR-IB and thus in this case BMPR-IA can functionally replace BMPR-IB. Furthermore, in this cell line the concentration for half-maximal ALP induction is about 10-fold lower than for BMP-2, which correlates very nicely with the difference in BMPR-IA affinity of both BMP factors (Nickel *et al.*, 2005). In contrast, the mouse osteoblastic cell line MC3T3 or the mouse myoblastic cell line C2C12, which express BMPR-IA but not BMPR-IB, do not respond to GDF-5 in the alkaline phosphatase expression assay (but at the same time respond to BMP-2) (Nishitoh *et al.*, 1996). Besides the fact that in the context of the developing joint BMPR-IA might not be the correct signaling receptor for GDF-5, the spatially highly defined expression pattern of GDF-5 and the two BMP type I receptors in

the junction between the growth plate and the developing joint suggests that at sites of high GDF-5 concentration only BMPR-IB is highly expressed whereas BMPR-IA expression is rather low (see Fig. 3) ((Wolfman *et al.*, 1997, Zou *et al.*, 1997, Sakou *et al.*, 1999, Storm & Kingsley, 1999, Yi *et al.*, 2001, Settle *et al.*, 2003, Minina *et al.*, 2005) for review see (Pogue & Lyons, 2006)).

All BDA2 causing BMPR-IB mutations are located in the cytoplasmic kinase domain. One exchange - isoleucine 200 to lysine (I200K) - is placed within the so-called GS (glycine/serine-rich) box, which is phosphorylated upon ligand binding and hetero-oligomerization of the type I and type II receptors (see Fig. 6A-C). Structural analysis of the kinase domains of the

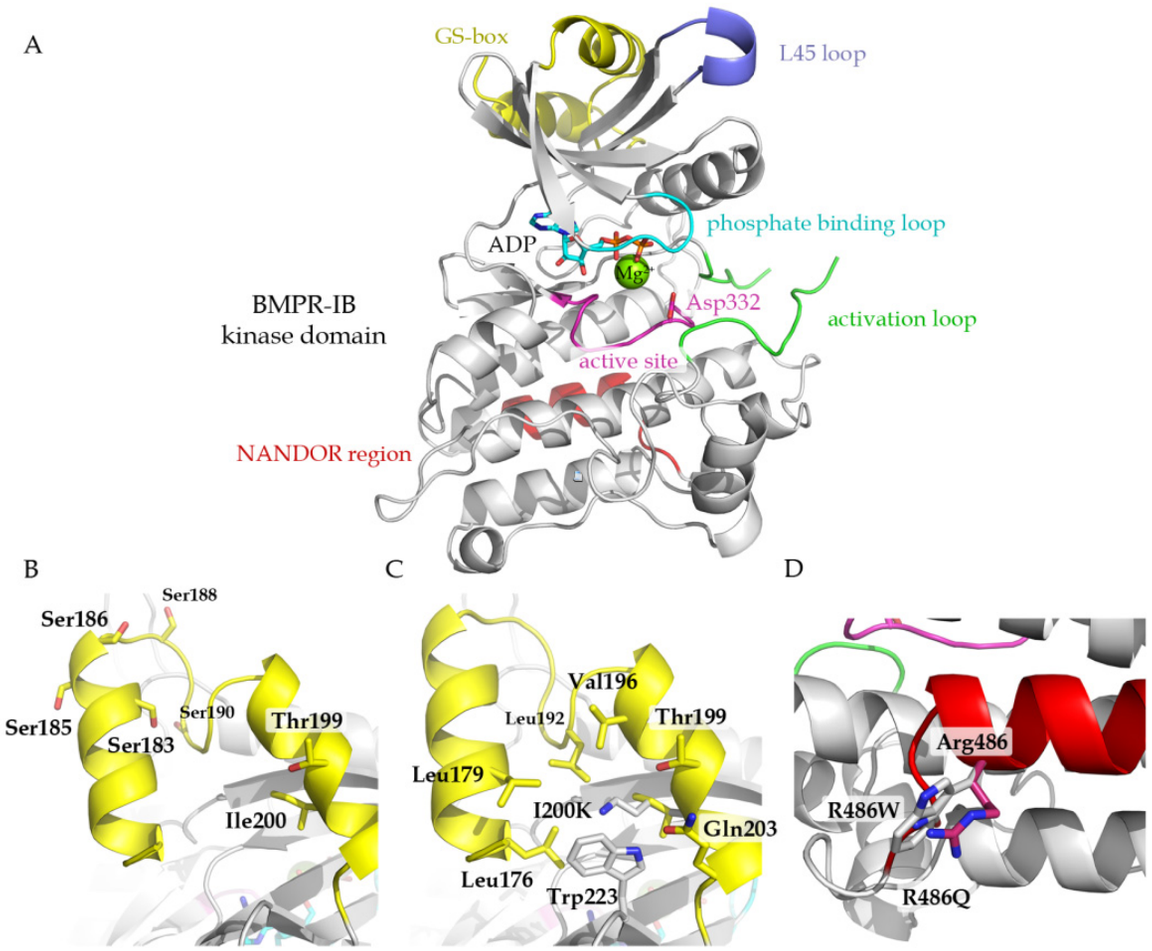

Figure 6. The kinase domain of the BMP receptor IB. A) Ribbon representation of a model of the BMPR-IB kinase domain (adapted from PDB entry 3MDY, (Chaikuad *et al.*, 2010a)). The elements important in kinase activity and or BMP signaling are indicated. Glycine/serine-rich (GS-)box: yellow; L45-loop for SMAD subgroup specificity: purple; phosphate binding loop: cyan; activation loop: green; active site with Asp332 in stick representation: magenta; NANDOR-region regulation downstream signal activation: red. B) Magnification of the GS-box with the relevant serine and threonine residues that become phosphorylated during BMP type I receptor activation shown as sticks. The location of Ile200 mutated in BDA2 is indicated. C) Isoleucine 200, mutated to lysine in BDA2, is surrounded by hydrophobic residues. Threonine 199, which is required to become first phosphorylated to allow for further phosphorylation events in the GS-box, is located in close proximity, suggesting that mutation

I200K might also act via abrogating the initial activating phosphorylation at Thr199. D) Magnification into the NANDOR domain of BMPR-IB. The mutated residue Arg486 is located at the solvent-accessible surface, thus mutations R486W and R486Q (shown in grey) very likely do not cause conformational alterations. This suggests that the NANDOR domain constitutes a binding interface for so far unknown proteins involved in the receptor activation.

BMP receptor BMPR-IB (PDB entry 3MDY, (Chaikuad *et al.*, 2010a)), of the TGF-β receptor TGFβR-I (Huse *et al.*, 1999) or the Activin type I receptor ActR-I (PDB entry 3H9R, (Chaikuad *et al.*, 2010b)) show that the GS-box domain in the inactivated state consists of two antiparallel α-helices. Functional analysis of the TGFβR-I receptor kinase revealed that phosphorylation of all conserved serine and threonine residues in the consensus motif (T/S)SGSGSG placed in the loop between the two helices is absolutely required for downstream signaling (Wieser *et al.*, 1995) and SMAD protein binding (Huse *et al.*, 2001). More importantly, threonine residue Thr200 in TGFβR-I (equivalent to Thr199 in BMPR-IB) adjacent to this consensus motif is absolutely conserved between TGF-β type I receptors and is crucial for ligand-dependent receptor activation. Mutagenesis showed that phosphorylation of this particular threonine residue is a pre-requisite for further phosphorylation of the GS-box motif located N-terminally of this residue (Wieser *et al.*, 1995). In the BDA2 associated mutation I200K in BMPR-IB the direct neighbor of Thr199 is exchanged from a hydrophobic isoleucine to a polar lysine residue. As the isoleucine is rather buried in this motif, the exchange might lead to local unfolding or the Ile to Lys substitution is such drastic that the recognition by the kinase responsible for phosphorylation of Thr199 and thus subsequent receptor activation is impeded (see Fig. 6A-C). *In vitro* kinase assays indeed revealed a complete loss of kinase activity of BMPR-IB carrying the I200K mutation (Lehmann *et al.*, 2003).

The other mutations in BMPR-IB associated with BDA2, R486Q or R486W, are located in the so-called NANDOR region (for non-activating non-down-regulating) (see Fig. 6A/D). This region at the C-terminus of the kinase domain is highly conserved between TGF-β type I receptors but placed quite distantly from the regulatory important regions such as the GS-box or the L45-loop, which mediate binding to R-SMAD proteins upon receptor activation or the active site of the kinase domain. Studies on the TGF-β receptors TGFβR-I (Garamszegi *et al.*, 2001) and TSR-I (Alk1) (Ricard *et al.*, 2010) show that mutations within this domain abrogate type I receptor endocytosis and signal transduction as R-SMAD proteins are not phosphorylated by these receptor mutants. In BMPR-IB the exchange of the surface-accessible arginine 486 by either glutamine or tryptophan diminished not only SMAD1/5/8 phosphorylation, but also led to strongly decreased expression of alkaline phosphatase in C2C12 cells transfected with BMPR-IB. This signaling-impaired phenotype could also be confirmed in a more physiological assay measuring chondrocyte differentiation in virally transduced chicken limb-bud micromass cultures (Lehmann *et al.*, 2003, Lehmann *et al.*, 2006). The effects of these mutations on downstream SMAD-dependent and SMAD independent signaling pathways as well as receptor endocytosis suggests that this region likely constitutes a binding site for not yet identified signaling components required for general receptor activation.

Skeletal malformation diseases have also been linked to mutations in the BMP signaling modulator Noggin, which directly binds to various BMP as well as GDF ligands and, when harboring mutations interfering with ligand binding, can cause skeletal malformations of the brachydactyly type. Noggin initially identified as a dorsalizing factor expressed in the Spemann organizer (Smith & Harland, 1992) was found to be an efficient BMP antagonist, which - by binding to the BMP ligands in the extracellular space with extremely high affinity in the picomolar range - can completely abrogate receptor binding and thus BMP signaling (Holley *et al.*, 1996, Zimmerman *et al.*, 1996). Despite its role in establishing a long-range BMP-4 morphogen gradient for dorsal-ventral patterning during gastrulation, Noggin also has functions later in development of the embryo (for a recent review see (Krause *et al.*, 2011)). Noggin knockout mice are embryonically lethal and show a complex phenotype (McMahon *et al.*, 1998), however it is important to note that mice being heterozygous for the Noggin null mutation develop normally (Brunet *et al.*, 1998). This suggests that the defects seen upon Noggin deletion do not result from gene dosage effects. Due to its expression in the ectoderm, loss of Noggin resulted in a severe neural tube phenotype with a failure of neural tube closure and a dramatic reduction in the amount of posterior neural tissue. As Noggin seems essential for ventral cell fates in the CNS development, motor neurons and ventral interneurons were lacking (McMahon *et al.*, 1998). Besides the neural abnormalities Noggin knockout mice showed also a drastically altered skeletal development (Brunet *et al.*, 1998, Tylzanowski *et al.*, 2006). All skeletal elements are affected with the severity of the axial defects increasing towards the posterior direction. However, analysis for ossification shows that the time point for ossification in these elements seems unchanged. These observations suggest that the loss of Noggin in the knockout mice affects cartilage development. The ablation of Noggin also affects limb development, with null mice having shorter limbs and fusions of various joints. By the use of a heterozygous transgene, where the Noggin gene has been replaced by *lacZ*, expression of Noggin in the developing limb could be analyzed in detail (Brunet *et al.*, 1998), showing that Noggin is strongly expressed in cartilage zones later forming bone, but is expressed at low levels or is absent in hypertrophic cartilage or joint cavities where GDF-5 expression is usually high. Analysis of the *NOG*$^{-/-}$ mice shows a massive overgrowth of cartilage in the limb, indicating that in wildtype mice Noggin represses the growth of these tissues in a negative feedback loop manner. It is known that in addition to GDF-5 a number of other BMPs, e.g. BMP-2, BMP-4, BMP-6 and BMP-7 are expressed in the limb and even the developing joints (Lyons *et al.*, 1989, Brunet *et al.*, 1998). Differential signaling of these different BMPs is required to induce apoptosis in interdigital tissues (Macias *et al.*, 1997) and in *Drosophila* sharp zones of activity of the fly BMP-homolog DPP, which do not necessarily correlate with the local DPP concentration, trigger local cell death to define joints (Manjon *et al.*, 2007). The locally highly variable expression of Noggin in the developing limb could provide for such a BMP activity modulating mechanism as *in vivo* Noggin inhibition of BMP signaling has distinct BMP specificity profiles (Zimmerman *et al.*, 1996, Seemann *et al.*, 2009, Song *et al.*, 2010). The important regulatory role of Noggin as an BMP antagonist is also highlighted by the fact that the Noggin gene is a mutational hotspot in several skeletal malformation diseases of the brachydactyly type BDB as well as the more severe multiple synostosis syndrome (SYNS1),

proximal symphalangism (SYM1), tarsal-carpal coalition (TCC) or SABTT (stapes ankylosis with broad thumbs and toes) syndromes (for a recent review see (Potti *et al.*, 2011)).

3.3. Noggin a BMP interacting hub during limb and joint formation

Structure analysis of the complex of BMP-7 bound to Noggin provided insights into the molecular mechanism how Noggin antagonizes BMP signaling (Groppe *et al.*, 2002). The homodimeric Noggin embraces the BMP ligand and simultaneously blocks type I and type II receptor binding via its C-terminal four-stranded β-sheet structure resembling a finger-like structure as found in BMPs itself and a N-terminal peptide segment called clip (see Fig. 7). Whereas the type II receptor-binding epitope of BMP-7 is blocked by the large and structured C-terminal part, type I receptor binding is only inhibited by the small clip segment (Gln28 to Asp39 of human Noggin). Very few polar interactions, mainly between the polar main chain atoms of the Noggin clip and residues from BMP-7, stabilize this interaction. In addition to the polar interactions, Pro35 of Noggin, which is found mutated in several skeletal malformation diseases (Gong *et al.*, 1999, Dixon *et al.*, 2001, Mangino *et al.*, 2002, Lehmann *et al.*, 2007, Hirshoren *et al.*, 2008), points into a hole in the type I receptor-binding epitope of BMP-7 formed by hydrophobic residues thereby mimicking a key interaction in the BMP ligand-type I receptor interaction (Hatta *et al.*, 2000, Kirsch *et al.*, 2000, Kotzsch *et al.*, 2009).

The disease-causing mutations in Noggin known today can be clustered into three regions: the mutations located in the clip (P35A/S/R, A36P, P37R, P42A/R; (Gong *et al.*, 1999, Dixon *et al.*, 2001, Mangino *et al.*, 2002, Debeer *et al.*, 2004, Lehmann *et al.*, 2007, Hirshoren *et al.*, 2008, Oxley *et al.*, 2008)), the β-sheet domain (E48K, P42A;P50R, R167G, L203P, R204L, W205C, W217G, I220N, Y222D/C, and P223L; (Gong *et al.*, 1999, Dixon *et al.*, 2001, Takahashi *et al.*, 2001, Kosaki *et al.*, 2004, van den Ende *et al.*, 2005, Weekamp *et al.*, 2005, Dawson *et al.*, 2006, Lehmann *et al.*, 2007, Oxley *et al.*, 2008, Emery *et al.*, 2009)) or the dimerization domain (C184Y, P187S, G189C, M190V, and C232Y; (Gong *et al.*, 1999, Takahashi *et al.*, 2001, Lehmann *et al.*, 2007, Oxley *et al.*, 2008, Rudnik-Schoneborn *et al.*, 2010)). The molecular mechanisms by which these mutations disrupt proper function of Noggin can be classified in part. Mutations of prolines or from other residues to proline, e.g. P42R, P50R, P187S, L203P, or P223L, will potentially lead to misfolding of the Noggin mutant, such that local structures cannot be maintained leading to a secondary loss of other Noggin-BMP interactions or to lower dimer stability (and hence to decreased secretion) if these exchanges occur in the dimerization domain (see Fig. 7) (e.g. P187S, (Lehmann *et al.*, 2007)). Some mutations in Noggin involving proline residues and occurring in the clip region disrupt BMP-Noggin hydrogen bonds, e.g. A36P, P37R or introduce steric hindrance by replacing the proline residue for geometrically non-fitting amino acids, e.g. P35A, P35S, or P35R. Various amino acid exchanges observed in the β-sheet domain substituting a hydrophobic residue for a polar, e.g. I220N, or replacing a large hydrophobic amino acid in the hydrophobic core with a smaller one, e.g. W205C, W217G, Y222C, probably cause local unfolding and thus weaken the Noggin:BMP binding. The amino acid residues Glu48, Arg167 and Arg204 together form a hydrogen bond network, thus mutation of any of these

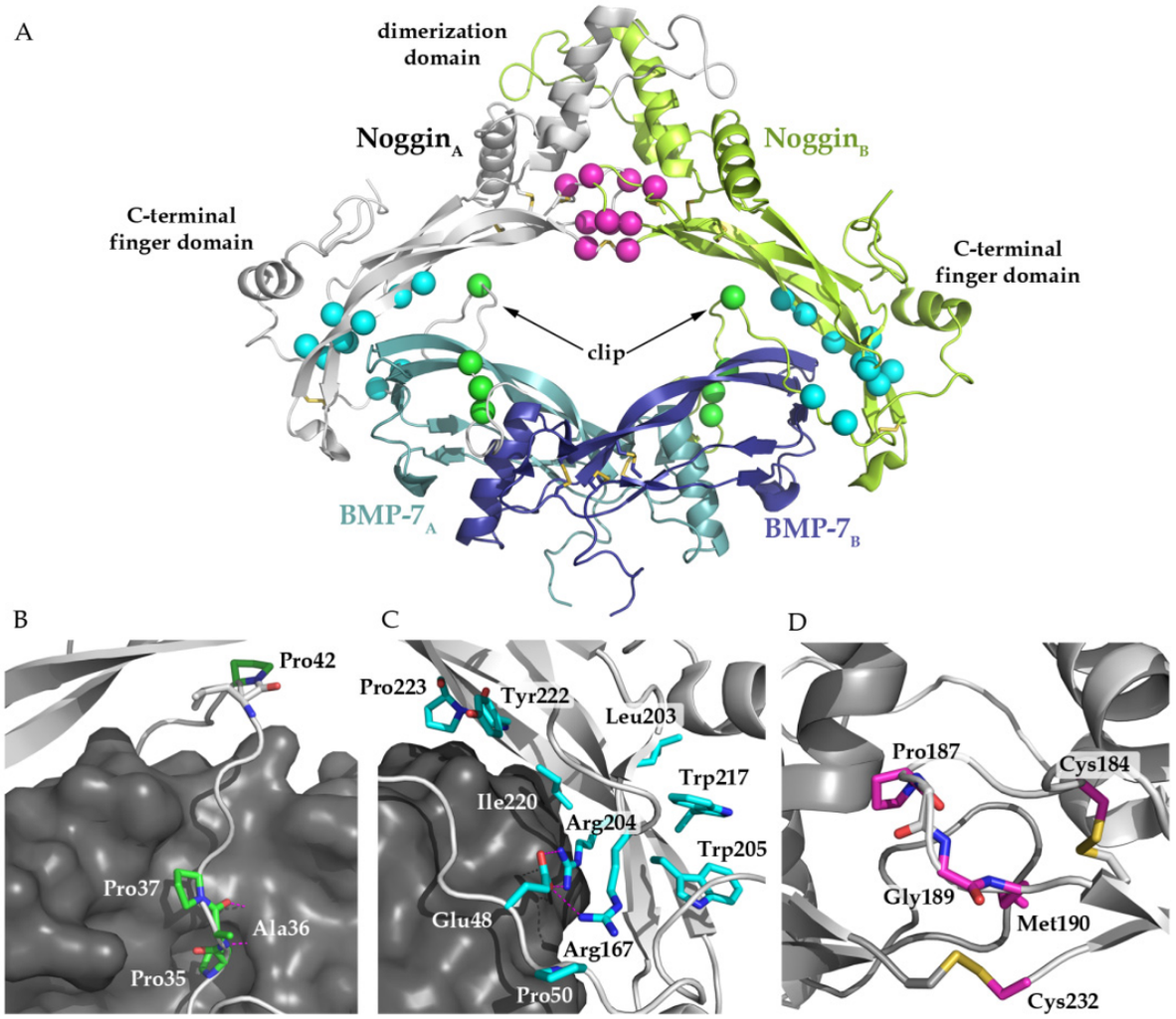

Figure 7. BMP inhibition by the modulator Noggin. A) Ribbon representation of the BMP-7:Noggin complex (PDB entry 1M4U, (Groppe *et al.*, 2002)). The dimeric Noggin (grey and light green) consists of three domains: the clip region located at the N-terminus, the C-terminal finger or β-sheet domain and a dimerization domain. By embracing the BMP ligand through the clip region and the C-terminal finger domain Noggin effectively blocks binding of type I and type II receptors thereby antagonizing BMP signaling. Mutations in Noggin identified in skeletal malformation diseases are shown as spheres color-coded according to their location in the aforementioned domains (green: clip region; cyan: finger/◎-sheet domain; magenta: dimerization domain). B) Magnification into mutationally affected interactions between residues of the Noggin clip region and BMP-7 (shown as grey van der Waals surface representation). Mutation of the indicated residues (Pro35, Ala36, Pro37, and Pro42 are shown as stick representations with C-atoms in green) likely alters the conformation of the Noggin clip or disrupts polar interactions (indicated by stippled magenta lines) between Noggin and BMPs. C) Magnification into the interface between the Noggin finger domain and BMP-7. Residues in Noggin involved in skeletal malformation diseases upon mutation are shown as sticks (C-atoms are colored in cyan). Most mutations likely affect local folding of the finger domain thereby attenuating or disrupting Noggin binding to BMPs. D) Magnification into the dimerization domain of Noggin. Residues involved in disease-causing mutations are shown as sticks with the C-atoms colored in magenta. Mutation of most of the residues displayed will likely interfere with dimerization of Noggin, e.g. mutation of either Cys184 or Cys232 will directly disrupt the intermolecular disulfide bond or possibly shuffle the disulfide bond pattern in the dimerization domain.

three residues will disrupt this network likely causing local structure changes in the β-sheet domain of Noggin. Furthermore, all three charged residues are buried upon binding to BMP ligands, thus mutations resulting in unbalanced charges will probably lead to electrostatic repulsion upon ligand binding. The mutations in Noggin's dimerization domain, e.g. C184Y, P187S, G189C, M190V, or C232W, all will very likely disturb efficient dimerization either by disrupting the intermolecular disulfide bond through the formation of non-native intramolecular disulfide pairs or through interfering with the homodimer interface (see Fig. 7D) (Marcelino *et al.*, 2001, Lehmann *et al.*, 2007).

Interestingly, mutations in Noggin represent a rather heterogeneous picture of skeletal malformations with different digits being affected and from a mild phenotype, e.g. BDB2 to more severe traits, e.g. SYM1 or SYNS1 (Lehmann *et al.*, 2007, Potti *et al.*, 2011). A direct correlation between the location of the mutation in Noggin and the severity of the malformation seems not apparent although mutations in the clip domain are diagnosed more frequently with BDB2 and mutations in the dimerization domain usually result in SYM1 or SYNS1 disease (Potti *et al.*, 2011). From a structural point of view these possible differences might be explained due to the fact that destabilizing changes in the clip region of Noggin might affect only certain BMPs. Analysis of *in vitro* binding of BMP-7 to the Noggin mutant P35R showed a rather small 7-fold decrease in BMP binding affinity (Groppe *et al.*, 2002). For BMPs that exhibit high affinities for their type I receptors, e.g. BMP-2, BMP-4 or GDF-5 the weakened binding of the clip of Noggin to these ligands might allow for a competition mechanism in which the receptor binding to a Noggin:BMP complex subsequently strips off the antagonist. For those BMPs that have low binding affinities to their type I receptors, e.g. BMP-5, BMP-6 and BMP-7 even the decreased binding of the Noggin clip to the ligand is still sufficient to block receptor binding and hence signaling of these BMPs. The mutations in the β-sheet region of Noggin, however, should affect all BMP ligands similarly and the severity of the phenotype should principally correlate with the loss of BMP binding affinity. The amino acid substitutions in the Noggin dimerization domain are expected to exhibit the strongest phenotype as these mutations strongly affect dimerization and secretion efficiency of the Noggin protein. Even if a monomeric Noggin variant protein might be secreted, its binding to BMPs as a monomer will be severely impaired due to the loss of avidity. Thus the mutations in the clip of Noggin might only affect a subset of the different BMPs present in the developing joint thereby causing a distinct phenotype, whereas the other Noggin mutations more likely resemble the phenotype of a Noggin null mutation. With respect to the direct effect of Noggin on GDF-5 it is important to note that in mice even though the strongest expression of *GDF5* mRNA is found in the joint, Noggin mRNA here is absent at these late stages of joint development. Thus it is unclear at which timepoints the BMP antagonist Noggin directly modulates GDF-5 during joint formation *in vivo* (Brunet *et al.*, 1998). Furthermore, it has been shown that the loss of Noggin in homozygous null mice leads to a strong downregulation of the *GDF5* mRNA message (Brunet *et al.*, 1998), which would be compatible with the observed effect in loss-of-function Noggin mutants.

3.4. GDF-5: A key molecule in joint development and maintenance

Besides Noggin, the *GDF5* gene has been identified as a mutational hotspot in skeletal malformation diseases. To date, 14 missense mutations as well as a multitude of frameshift mutations have been identified in the translated region of the *GDF5* gene. Furthermore single nucleotide polymorphisms (SNPs) in the 5′ and 3′ untranslated region of the *GDF5* gene, three of which could be linked to enhanced susceptibility of developing osteoarthritis (OA), suggest that tempero-spatially highly defined gene expression of GDF-5 is required throughout life and is not limited to limb and joint development during embryogenesis (see Table 1 and Fig. 8).

Two SNPs in the 5′ untranslated regions (UTR) of *GDF5*, rs143383 and further downstream rs143384, share both a T-to-C transition in the *GDF5* core promoter. Functional studies using RNA extracted from the articular cartilage of OA patients harboring the SNP rs143383 revealed a significant, up to 27% reduced expression level of the osteoarthritis-associated T-allele relative to the C-allele, a phenomenon termed differential allelic expression (DAE) (Southam *et al.*, 2007). This allelic expression imbalance of *GDF5* could be extended to other soft tissues of the whole synovial joint, emphasizing that the single nucleotide

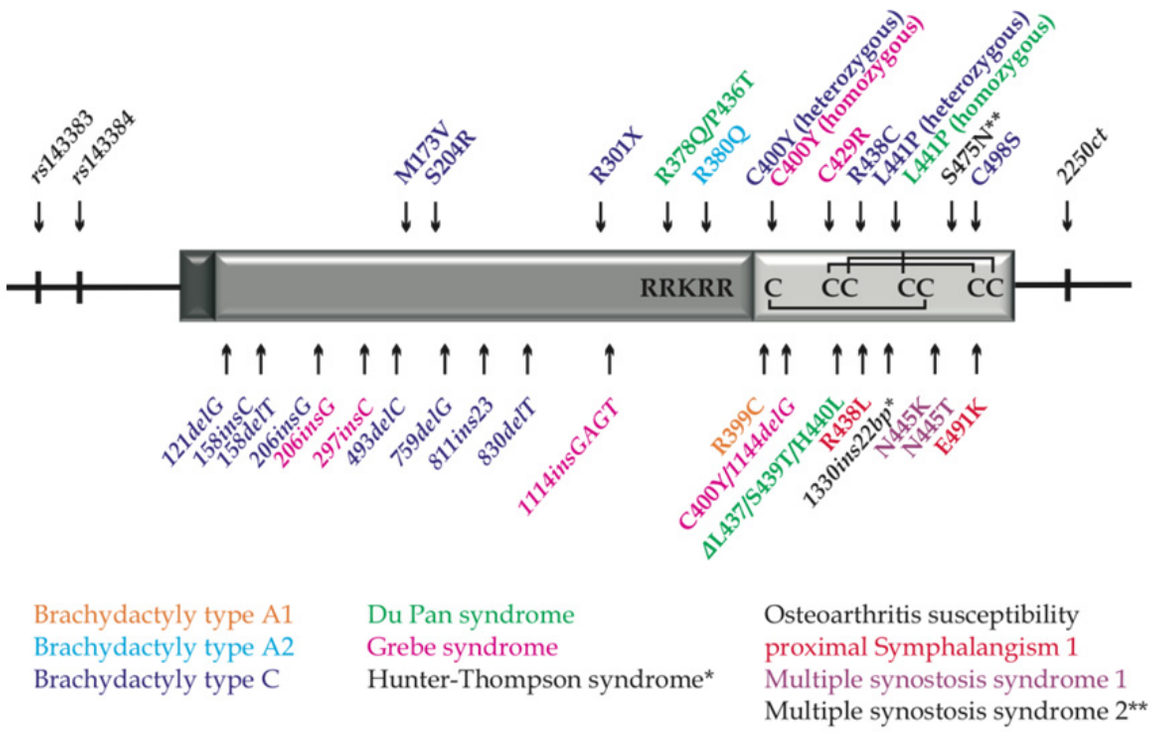

Figure 8. Localization of *GDF5* mutations. Arrowheads indicate the location of all currently known mutations linked to human skeletal malformation diseases affecting the limb. The specific inherited disease caused by each mutation is displayed in the legend underneath.
A GDF-5 monomer consists of an N-terminal signal peptide domain (black box), a prodomain (dark grey box) and the C-terminal mature part (light grey box) containing six highly conserved cysteine residues forming the cystine knot motif, whereas the seventh cysteine connects two monomers via an intermolecular disulfide bond. Italic type indicates nucleotide nomenclature; normal type represents single amino acid nomenclature. For references see Table 1.

mutation	location	hetero-/homozygous	disease	OMIM #	reference
rs143383	5´UTR *gdf5* gene	heterozygous	Osteoarthritis susceptibility	# 612400	(Miyamoto *et al.*, 2007)
rs143384	5´UTR *gdf5* gene	heterozygous	Osteoarthritis susceptibility	# 612400	(Rouault *et al.*, 2010)
2250ct	3´UTR *gdf5* gene	heterozygous	Osteoarthritis susceptibility	# 612400	(Egli *et al.*, 2009)
121delG	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Polinkovsky *et al.*, 1997)
158delT	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Everman *et al.*, 2002)
158insC	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Everman *et al.*, 2002)
206insG	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Polinkovsky *et al.*, 1997)
206insG	prodomain *gdf5* gene	homozygous	Chondrodysplasia, Grebe type	# 200700	(Stelzer *et al.*, 2003)
297insC	prodomain *gdf5* gene	homozygous	Chondrodysplasia, Grebe type	# 200700	(Faiyaz-Ul-Haque *et al.*, 2002a)
493delC	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Galjaard *et al.*, 2001)
M173V	prodomain *gdf5* gene	homozygous	Brachydactyly type C	# 113100	(Schwabe *et al.*, 2004)
S204R	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Everman *et al.*, 2002)
759delG	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Polinkovsky *et al.*, 1997)
811ins23	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	Everman, D. B. et al. 2002)
830delT	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	Everman, D. B. et al. 2002)
R301X	prodomain *gdf5* gene	heterozygous	Brachydactyly type C	# 113100	(Polinkovsky *et al.*, 1997)
1114insGAGT	prodomain *gdf5* gene	homozygous	Chondrodysplasia, Grebe type	# 200700	(Basit *et al.*, 2008)
R378Q/P436T	prodomain *gdf5* gene; processing site / mature domain	compound heterozygous	Acromesomelic dysplasia, DuPan syndrome	# 601146	(Douzgou *et al.*, 2008)
R380Q	prodomain *gdf5* gene; processing site	heterozygous	Brachydactyly type A2	# 112600	(Ploger *et al.*, 2008)
R399C	mature domain	heterozygous	Brachydactyly type A1	# 112500	(Byrnes *et al.*, 2010)
C400Y	mature domain; no processing/secretion	heterozygous	Brachydactyly type C	# 113100	(Thomas *et al.*, 1997)
C400Y	mature domain; no processing/secretion	homozygous	Chondrodysplasia, Grebe type	# 200700	(Thomas *et al.*, 1997)
C400Y/*del1144G*	mature domain/	compound	Chondrodysplasia,	# 200700	(Thomas *et*

	prodomain; no processing/secretion	heterozygous	Grebe type		*al.*, 1997)
mW408R (hW414R)	mature domain; location in type I receptor binding site	heterozygous	Brachypodism		(Masuya *et al.*, 2007)
mW408R (hW414R)	mature domain; location in type I receptor binding site	homozygous	severe Brachypodism, Osteoarthritis		(Masuya *et al.*, 2007)
C429R	mature domain	homozygous	Chondrodysplasia, Grebe type	# 200700	(Faiyaz-Ul-Haque *et al.*, 2008)

Table 1. Table of all known mutations in *GDF5* gene linked to skeletal malformation diseases affecting the limb. Mutations depicted in red represent single nuclear polymorphisms (SNPs) located in 5′ or 3′ regulatory regions of *GDF5* gene. Shown in black are mutations situated in the prodomain, whereas mutations in the mature part are represented in blue. Frameshift mutations are highlighted in italics, non-sense mutations are underlined.

polymorphism is not restricted to cartilage (Egli *et al.*, 2009). In addition, recent analysis showed that expression of GDF-5 could be further modulated epigenetically as both C-alleles of the SNPs rs143383 and rs143384 form CpG sites thereby explaining the intra- and inter-individual variations observed (Reynard *et al.*, 2011). A third SNP influencing GDF-5 expression, 2250ct, is found in the 3′ UTR of *GDF5*. It acts independently from the 5′ SNP rs143383 and can similarly reduce protein expression levels by 20-25% (Egli *et al.*, 2009). The independent reduction in expression by these SNPs can be additive thereby showing that even moderate imbalances in the allelic expression levels of *GDF5* can result in severe disturbances in synovial joint maintenance. This idea is further emphasized by the identification of a duplication in the 3′ UTR of the *BMP2* gene including a distant enhancer of BMP2 expression in BDA2 patients. The phenotype described by Dathe *et al.* resembles those caused by specific mutations in the *GDF5* or the *BMPR1B* gene (Dathe *et al.*, 2009). As BMP-2 is expressed in regions surrounding future joints as well as in the joint interzone during the development of interphalangeal joints in close proximity to GDF-5 expression, one could hypothesize that by either increasing BMP-2 levels due to the duplication of an enhancer or by decreasing the GDF-5 expression due to regulatory SNPs as described above, the fine-tuned balance between signals from different BMPs may be severely disturbed.

3.5. Proper folding and processing of pro-GDF-5 is essential for GDF-5 signaling

Like other ligands of the TGF-β superfamily GDF-5 is expressed and secreted as a dimeric pro-protein consisting of a large (354aa per monomer) pro-part and a smaller (120aa per monomer) mature part at the C-terminus. The C-terminal mature part harbors the characteristic motif present in all TGF-β ligands comprising of seven (BMPs, GDFs) highly conserved cysteine residues (Activins, TGF-βs have two further Cys residues at the N-terminus of the mature part) of which six form the so-called cystine knot. The seventh cysteine residue is involved in an intermolecular disulfide bond thereby stabilizing the (usually homo-)dimeric ligand assembly. The dimeric mature part of TGF-β ligand exhibits

a butterfly shaped assembly with the monomeric subunits adopting an architecture resembling a left hand (Sebald *et al.*, 2004). The dimer interface is formed by the palm of the hand, two two-stranded β-sheets resembling two fingers emanate from the cystine knot containing palm. Mutagenesis was used to determine the receptor binding epitopes (Kirsch *et al.*, 2000). The BMP type I receptors bind to the so-called wrist epitope, the type II receptors bind to the so-called knuckle epitope (Kirsch *et al.*, 2000). The location of these receptor binding epitopes were then confirmed by structure analyses of various BMP ligand-receptor complexes (Kirsch *et al.*, 2000, Greenwald *et al.*, 2003, Allendorph *et al.*, 2006, Weber *et al.*, 2007, Kotzsch *et al.*, 2009).

Homozygous non-sense or frame-shift mutations in the pro- or mature part of *GDF5* will result in a complete knockout of *GDF5*. However, also heterozygous non-sense and frame-shift mutations in *GDF5* will severely lower the level of intact protein; assuming equal transcriptional and translational efficiency from both alleles by statistics only 25% of the protein produced will be intact due to its dimeric nature. Hence the complete knockout or partial knockdown of *GDF5* achieved by this type of mutation leads to rather severe skeletal malformation phenotypes such as brachydactyly type C (BDC), symphalangism (SYM1) or multiple synostosis syndrome (SYNS1). One potentially underappreciated possibility is also the formation of nonfunctional heterodimeric ligands if a cell produces more than one TGF-β factor at a time and thus a possible influence of non-sense GDF-5 mutations onto other BMP signals. It is a known fact that in Drosophila the BMP-2 and BMP-7 orthologs Dpp and Screw can form heterodimers with unique functions required for proper development of certain tissues (Shimmi *et al.*, 2005, O'Connor *et al.*, 2006), however in vertebrates existence of such BMP heterodimers has only been postulated or recombinant proteins have been used in the analysis, but existence of such heterodimers has not really been proven *in vivo* (Schmid *et al.*, 2000, Butler & Dodd, 2003) thus a potential "cross"-influence of non-functional GDF-5 mutations on other BMPs can only be hypothesized.

Of the 14 missense mutations known in the *GDF5* gene four are located within the pro-part of the GDF-5 protein. Whereas for the TGF-βs the pro-part fulfills an important regulatory role, termed latency, its role for the BMP and GDF subgroup of the TGF-β superfamily is much less clear. Latency was discovered for TGF-β1 in 1984 showing that TGF-β proteins are secreted as large protein complexes that require activation for TGF-β signaling (Lawrence *et al.*, 1984). It is known today that upon secretion the pro-part of TGF-βs is cleaved in the Golgi apparatus by furin proteases at a site between the pro- and mature part containing a consensus RXXR motif (other proteases might substitute for furin proteases but providing for TGF-β proteins with different N-termini) (Dubois *et al.*, 1995). The pro-part also called latency-associated peptide (LAP) however is still non-covalently attached thereby interfering with TGF-β signaling. Activation corresponding to release of the mature part from this intermediate latent complex is achieved either by physicochemical changes in the environment, e.g. acidification or by further proteolysis. Proteins specifically binding LAP have been identified (Miyazono *et al.*, 1988), these latent TGF-β binding proteins (LTBP) interact with the extracellular matrix and play an important role in the TGF-β activation process (for review see (Annes *et al.*, 2003)). For BMPs a process identical to latency as

observed for TGF-βs is not known, but the pro-part of the BMPs possibly enhances the otherwise poor solubility of BMPs under physiolocigal conditions and thus might provide for or enhance their long-range activity (Sengle *et al.*, 2008, Sengle *et al.*, 2011). Recent determination of the structure of the TGF-β1 pro-protein now provides for an insight in the regulatory mechanism of the pro-part at atomic level (Shi *et al.*, 2011). The pro-part embraces the mature part of TGF-β like a straitjacket, a long N-terminal α-helix binds into the type I receptor-binding site (in BMPs and GDFs called wrist epitope) thereby blocking receptor access to this epitope. A proline-rich loop termed latency lasso and a second α-helix encompass the fingertips and the back of the second finger of the mature part of TGF-β hence also blocking the type II receptor epitope. The pro-domain monomers form a dimerization site in the C-terminal region called bowtie, which is located above the butterfly-shaped dimeric TGF-β mature part. Two intermolecular disulfide bonds additionally stabilize the dimerization between the pro-domain subunits. Strikingly, the arrangement of the pro- and mature domain resembles the overall architecture found for the Noggin-BMP7 interaction (Groppe *et al.*, 2002). Both receptor-binding epitopes are tightly blocked from receptor access and the binding of the modulator/pro-domain is strongly enhanced through avidity by forming a covalently linked dimer. The importance of the covalent dimer linkage becomes obvious in the rare bone disorder Camurati-Engelmann disease in which these cysteine residues in the TGF-β1 pro-part are mutated resulting in a disrupted dimerization and leading to increased ligand activation (Janssens *et al.*, 2003, Walton *et al.*, 2010).

Although the sequence homology (as well as differences in the length) between the pro-domains of the various TGF-β members is certainly lower than between their mature parts alignments clearly show that all pro-domains will adopt a similar fold (Shi *et al.*, 2011). A homology model for pro-GDF-5 build on the basis of pro-TGF-β1 structure instantly provides for possible explanations to why the effect of latency is quite different between TGF-βs and members of the BMP subgroup. Particularly for GDF-5 (also true for GDF-6 and -7) many loops in the pro-domain are extended possibly creating further sites for proteolytic activation or degradation, secondly BMPs and GDFs lack the two cysteine residues present in the pro-domain being responsible for covalent linkage (see Fig. 9A). This suggests that the pro-domain association is much less stable for BMPs and GDFs (see mutations of cysteines in the Curati-Engelmann disease) and the release of the mature growth factor domain is facilitated without further need of processing. The four mutations in the GDF-5 pro-domain cluster in three different skeletal malformation phenotypes: M173V – BDC, S204R – BDC, R378Q/P436T (compound heterozygous) – Acromesomelic dysplasia, DuPan syndrome, R380Q – BDA2) indicating a loss-of-GDF-5 function in all cases (Everman *et al.*, 2002, Schwabe *et al.*, 2004, Douzgou *et al.*, 2008, Ploger *et al.*, 2008). On the basis of our own model methionine 173 is placed in close proximity to the first helix element blocking type I receptor binding, whereas serine 204 is placed in the so-called arm domain providing the structural scaffold for the straitjacket architecture. Both missense mutations likely lead to (local) unfolding and thus destabilize the pro-protein complex. This might subsequently lead to lower secretion efficiency and the observed loss-of-function phenotype. The mutation

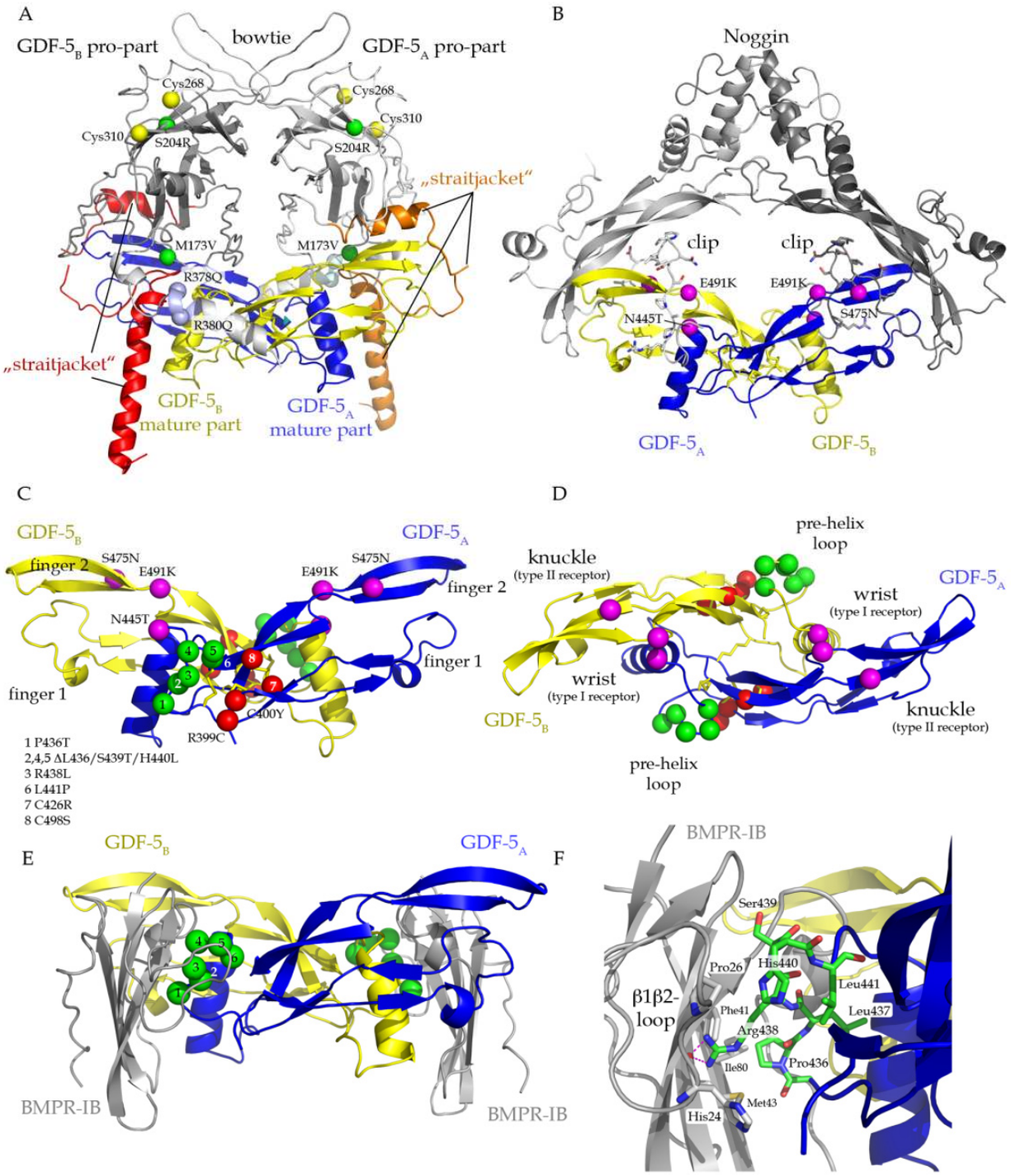

Figure 9. Mutations in GDF-5 and its effect on structure or interactions. A) Homology model of pro-GDF-5 based on the structure of pro-TGF-β1 in ribbon representation (Shi *et al.*, 2011). The mature part of GDF-5 (shown in blue and yellow) is embraced by the pro-part with the N-terminal part resembling a straitjacket (in red and orange). This element comprising of two helices block access to both type I and type II receptor binding epitopes. In contrast to the pro-part of TGF-βs the pro-domains of BMPs and GDFs likely do not have intermolecular disulfides (the potential positions of Cys268 and Cys310 are shown) suggesting that the pro/mature part assembly of BMPs and GDFs might be less stable compared to TGF-βs. Four missense mutations in the pro-part are found to be associated with skeletal malformation diseases: M173V, S204R, R378Q, and R380Q. The first two mutations (marked by green

spheres) possibly cause misfolding of the pro-domain thereby weakening the pro-protein and leading to lower secretion efficiency. The latter two mutations are located in the furin protease site (marked as light-blue spheres) and were shown to lower or abrogate proteolytic processing of the pro-protein. B) Homology model of the Noggin:GDF-5 complex (Schwaerzer *et al.*, 2011) based on the crystal structure of the Noggin:BMP-7 complex (Groppe *et al.*, 2002). Noggin, by a similar mechanism but different structural architecture, embraces GDF-5 thereby blocking receptor binding of either subtype through its clip and finger domains. Three missense mutations in GDF-5 associated with symphalangism were shown to have impaired GDF-5 – Noggin interaction: N445T/K, S475N, and E491K. All three mutations are in close proximity of the Noggin clip region suggesting that through loss of interaction with this element GDF-5 binding to Noggin is attenuated. C) Ribbon representation of the mature part of GDF-5 with the two monomeric subunits shown in blue and yellow. The architecture of a GDF-5 dimer resembles a left hand, the α-helix forming the palm, the two β-sheets depicting two fingers and the N-terminus marking the thumb. Consequentally, the receptor binding epitopes were named wrist (type I receptor), formed by the dorsal side of the fingers and the palm, and knuckle (type II receptor), formed by the ventral side of finger 1 and 2. The location of all known mutations associated with skeletal malformation diseases is depicted by spheres, with color-coding according to their belonging to either cystine knot mutations (red), pre-helix loop mutations (green) or mutations affecting Noggin-binding (magenta). D) As in C but rotated clockwise around the x-axis by 90°. E) Ribbon representation of the complex of GDF-5 (in blue and yellow) bound to the extracellular domain of BMPR-IB (grey). The overview clearly shows that affected residues in the pre-helix loop are in contact with receptor elements suggesting that these mutations alter type I receptor binding. F) Magnification of the interaction between residues in the pre-helix loop of GDF-5 and residues in the binding epitope of BMPR-IB. The complete pre-helix loop is tightly packed to residues in the threestranded β-sheet of BMPR-IB. GDF-5 Arg438 is involved in hydrogen bonds to His24 located in the β1β2-loop of BMPR-IB. The tight turn structures at the N- and C-terminal end of the pre-helix loop also indicate that the mutations involving the exchange of a proline (P436T) or introduction of a proline (L441P) will likely destroy the conformation of the pre-helix loop thereby affecting receptor binding even if these two residues do not form direct contacts with GDF-5.

R380Q targets the pro-domain cleavage site by destroying or attenuating proteolytic processing via furin proteases (Ploger *et al.*, 2008). The now covalent linkage of pro- and mature part of GDF-5 R380Q very likely enhances the competition of the pro-domain with receptor binding and thus leads to loss of or attenuated GDF-5 activity (Ploger *et al.*, 2008). The mechanism by which the double mutation R378Q/P436T causes the skeletal malformation is more complex. As the mutation is compound heterozygous, three GDF-5 variants are potentially produced in the patient. Statistically 50% of the GDF-5 protein would carry both exchanges as a heterodimer and the other 50% would consist of homodimers with either one of the two mutations. Heterozygous carriers of the individual missense mutations R378Q or P436T did not exhibit any skeletal phenotype thus preventing to point towards a particular mutation as disease-causing if found in a homozygous background. For the mutation R378Q it can be assumed that processing of the pro-protein is at least impaired and thus the portion of GDF-5 R378Q homodimer is likely to be inactive as found for R380Q (see Fig. 9) blank (Ploger *et al.*, 2008). The missense mutation P436T is located in the mature part of GDF-5 in the so-called pre-helix loop of the GDF-5 type I receptor-binding epitope (Nickel *et al.*, 2005). Mutation of the equivalent proline residue in BMP-2 strongly decreased binding of this BMP-2 variant to both type I receptors, BMPR-IA and BMPR-IB thus leading to a loss of BMP signaling (Kirsch *et al.*, 2000).

Of the other eight known disease-related amino acid exchanges in the mature part of GDF-5, several mutations involve the exchange of a cysteine residue participating in the formation of the cystine knot, e.g. C400Y, C429R, C498S or introduce additional cysteine residues, e.g. R399C, R438C, which will interfere with proper formation of the cystine knot, thereby leading to a misfolded inactive protein. Several studies show that under conditions mimicking a homozygous background no secretion of the GDF-5 variant is observed (Everman *et al.*, 2002, Dawson *et al.*, 2006). However, mutations involving cysteines can also act dominant-negatively (see Fig. 9). Thomas *et al.* tested the effect of the GDF-5 mutation C400Y, which is found homozygous in chondrodysplasia Grebe type (Thomas *et al.*, 1997). Upon transfection of only the mutated gene into COS-7 cells resembling a homozygous background no GDF-5 protein could be detected in the cell supernatant, however co-transfection of the genes for wildtype GDF-5 and the variant GDF-5 C400Y clearly attenuated GDF-5 protein levels in the supernatant. This effect was dose-dependent indicating that for heterozygous carriers through differential allelic expression a highly variable phenotype could possibly be observed (Thomas *et al.*, 1997). Furthermore, this study also indicated that the mutation might act dominant negative onto other BMPs by selective heterodimerization. By co-transfection of the gene encoding for GDF-5 C400Y together with either BMP-2, BMP-3 or BMP-7, heterodimers could be isolated from the cell supernatant that will most likely be non-functional (Thomas *et al.*, 1997).

3.6. GDF-5 activity is tightly regulated by the BMP antagonist Noggin

All other missense mutations in the *GDF5* gene cluster in two regions of the GDF-5 structure (see Fig. 9C/D). Three missense mutations cluster in close proximity of finger 2 of GDF-5, N445T/K (Seemann *et al.*, 2009), S475N (Akarsu *et al.*, 1999, Schwaerzer *et al.*, 2011) and E491K (Wang *et al.*, 2006). The heterozygous mutations N445T and N445K in GDF-5 were identified in patients suffering from multiple synostosis syndrome (SYNS1) characterized by fusion of carpal bones and proximal symphalangism in fingers II to V (Seemann *et al.*, 2009). Analysis of the recombinant GDF-5 variant in BMPR-IB transfected myoblastic C2C12 cells indicated that the mutation did not lead to a loss of GDF-5 function. In fact analyzing the expression of the osteogenic marker alkaline phosphatase in non-transfected C2C12 cells revealed even a gain of activity exemplified by a small but measureable ALP induction when stimulating with GDF-5 N445T but no induction of ALP expression when using wildtype GDF-5. As this activating mutation is located within the wrist (type I receptor binding) epitope of GDF-5 differences in binding to the BMP type I receptors were assumed. However, competition assays using soluble receptor ectodomains showed that binding of the GDF-5 variant N445T to BMPR-IA as well as BMPR-IB is unaltered (Seemann *et al.*, 2009). Sequence comparison with other BMP factors indicated that one of the mutations found, the exchange of Asn445 to lysine, is native in BMP-9 and BMP-10. As the latter factors are insensitive to Noggin inhibition, Seemann *et al.* assumed that this mutation also renders GDF-5 insensitive to inhibition by Noggin. *In vitro* assays indeed confirmed that GDF-5 N445T is not antagonized by recombinant Noggin protein leading to an increase in GDF-5 signaling activity during early stages of limb and joint development where Noggin and *GDF5* expression patterns overlap (Seemann *et al.*, 2005, Seemann *et al.*, 2009). Another mutation in GDF-5 leading to proximal symphalangism is

E491K discovered in two large Chinese families (Wang *et al.*, 2006). The skeletal malformation phenotype resembles the one seen in aforementioned patients having either the mutation N445T/K (Seemann *et al.*, 2009) or R438L (Seemann *et al.*, 2005) in the *GDF5* gene. Nothing is known about receptor or modulator protein binding of this particular GDF-5 variant, however in the GDF-5 structure Glu491 is in close proximity to Asn445. Moreover, the sidechain carboxamide group of Asn445 is forming a hydrogen bond to the backbone carbonyl of Glu491 possibly suggesting a similar disease-causing molecular mechanism through the loss of inhibition by Noggin as described above by Seemann *et al.* (2009). Modeling of a GDF-5:Noggin complex based on the structure of the BMP-7:Noggin interaction (Groppe *et al.*, 2002) does however not indicate a direct interference of a GDF-5:Noggin interaction by exchanging Glu491 by lysine (see Fig. 9).

The mutation S475N is another mutation in the mature part of GDF-5, which causes multiple synostosis syndrome (SYNS1), a phenotypic description of these heterozygous missense mutations was first reported by Akarsu *et al.* (1999). The phenotype again suggests a gain-of-function in GDF-5 signaling. A detailed analysis of the signaling properties of this GDF-5 variant indeed revealed that GDF-5 S475N is significantly more potent in the chondrogenic differentiation in chicken micromass culture compared to wildtype GDF-5 (Schwaerzer *et al.*, 2011). The mutation is located in the knuckle (type II receptor) epitope of GDF-5 (see Fig. 9C/D). Although no direct structural data is currently available for GDF-5 bound to type I and type II receptors, structure data available on ternary complexes of BMP-2 (Allendorph *et al.*, 2006, Weber *et al.*, 2007) indicated that this highly conserved serine residue is at the center of the BMP/GDF type II receptor interaction. Despite its location exchange of this residue in BMP-2 affected type II receptor binding only marginally (Weber *et al.*, 2007) suggesting that other residues in the BMP-type II receptor interface are more important for the ligand-receptor interaction. However, in GDF-5 Ser475 seems more important for the binding of BMPR-II as indicated by a 7-fold decrease in the binding affinity upon mutation to asparagine, which seems surprising given the fact that this mutant shows an elevated activity compared to wildtype GDF-5 (Schwaerzer *et al.*, 2011). As the BMP type II receptor epitope overlaps heavily with that of Noggin, also the change in binding to Noggin was determined showing that also Noggin binding affinity is similarly decreased by 4-fold. When the effect of Noggin inhibition on BMP factors was investigated by analyzing BMP-induced alkaline phosphatase expression or chondrogenic differentiation in chicken micromass culture in the presence of Noggin, GDF-5 S475N was clearly resistant to antagonizing effects by Noggin, whereas signals from wildtype GDF-5 could be efficiently blocked with Noggin (Schwaerzer *et al.*, 2011). This possibly indicates that the loss in BMP type II receptor binding affinity seen for this variant is overcompensated by the deprivation of Noggin-mediated inhibition (Schwaerzer *et al.*, 2011).

3.7. Type I receptor binding as well as receptor specificity is essential for correct GDF-5 function

A clear hotspot for disease-related mutations is found for the so-called pre-helix loop located in the wrist epitope of GDF-5 (Nickel *et al.*, 2005). This loop is the key interaction element for BMP-type I receptor interaction (Kirsch *et al.*, 2000, Keller *et al.*, 2004, Kotzsch *et*

al., 2008). For BMP-2 and GDF-5 this segment contains the so-called main binding determinant a highly conserved leucine residue, whose polar main chain atoms makes a pair of hydrogen bonds with a conserved glutamine residue present in the BMP type I receptors IA and IB. Mutation of either the leucine to a proline in BMP-2 or GDF-5 or the glutamine residue in BMPR-IA or BMPR-IB leads to a strongly reduced type I receptor affinity (Keller *et al.*, 2004, Kotzsch *et al.*, 2009). In the unbound state this pre-helix loop segment is also rather flexible allowing for geometrical adaptability to different receptor surface geometries. This observation together with the disordered and flexible ligand-binding epitope seen in the BMP type I receptors provides a mechanism for the pronounced ligand-receptor promiscuity seen in the BMP/GDF-subgroup of the TGF-β superfamily (Keller *et al.*, 2004, Allendorph *et al.*, 2007, Klages *et al.*, 2008, Kotzsch *et al.*, 2008, Saremba *et al.*, 2008). Despite structural analyses showed that the pre-helix is flexible before receptor binding, the mutation L441P suggests that in the bound state a geometrically defined conformation is required for (high affinity) binding of BMP type I receptors (Kotzsch *et al.*, 2009). Residue Leu441 is located at the C-terminal end of the pre-helix loop forming a sharp turn together with Ser439 and His440 (see Fig. 9E/F). The sidechain of Leu441 is oriented into the interior of GDF-5 making it implausible that its exchange to proline affects type I receptor binding through altering direct interactions. However, the different backbone torsion angle restraints of a non-proline compared to a proline residue suggest that the L441P mutation alters the conformation of the C-terminal end of the pre-helix loop and that hereby important non-covalent interactions between GDF-5 and its type I receptors are strongly impaired. Although earlier reports claim that the mutation L441P in GDF-5 affects binding to the BMP receptor IB (Faiyaz-Ul-Haque *et al.*, 2002b, Seemann *et al.*, 2005) our own data shows that binding to both BMP type I receptors is strongly attenuated (Kotzsch *et al.*, 2009). A rather complex mutation discovered by Szczaluba *et al.* in patients suffering from DuPan syndrome shows shortening of all toes as well as all fingers but the thumb (Szczaluba *et al.*, 2005). Here in the GDF-5 protein residue Leu437 is deleted and the adjacent residues Ser439 and His440 are mutated to threonine and leucine respectively (see Fig. 9). As these changes grossly alter the sequence as well as conformation of the pre-helix loop, it is not surprising that this GDF-5 compound variant shows no type I receptor binding at all (Kotzsch *et al.*, 2009). Interestingly, although the mutation was found to be heterozygous in the carrier it has a dominant-negative effect (Szczaluba *et al.*, 2005). Misfolding of the mutant protein and hence impaired secretion can be excluded as explanation, as the protein could be recombinantly produced and exhibits wildtype-like affinity to BMP type II receptors. One possible explanation for the quite strong skeletal phenotype might be that this GDF-5 variant is not only inactive but possibly still retains its Noggin-binding capability and therefore can act as a Noggin scavenger similar as to what was described for the BMP-2 variant L51P (Keller *et al.*, 2004).

The probably most interesting mutation in GDF-5 is the exchange of Arg438 to leucine found in patients suffering from proximal symphalangism (Seemann *et al.*, 2005). Based on a structural-function analysis to determine the GDF-5 type I receptor specificity this amino acid position – 438 if the complete pre-pro-protein is considered and position 57 if

numbering starts with the mature part of GDF-5 - was shown before to be solely responsible for the BMPR-IB binding preference of GDF-5 (see Fig. 9E/F) (Nickel *et al.*, 2005). The equivalent residue in BMP-2, which binds both BMP type I receptors, BMPR-IA and BMPR-IB, with equally high affinity is alanine. In contrast, in GDF-5 this position is occupied by a large positively charged arginine being also the largest difference in amino acid sequence within the central type I receptor-binding epitope. Upon exchange of Arg438 in GDF-5 to alanine, GDF-5 R438A bound both type I receptors with the same affinity and with binding characteristics indistinguishable from those of BMP-2 (Nickel *et al.*, 2005). Recent structure analysis of GDF-5 bound to its type I receptor BMPR-IB revealed a molecular mechanism by which GDF-5 "discriminates" between both type I receptors (Kotzsch *et al.*, 2009). A loop between the two N-terminal β-strands of the BMP type I receptors can adopt different conformations dependent on the amino acid sequence. As this loop is in contact to the "GDF-5 specificity determining" amino acid Arg438 BMP type I receptors can be selected through the presence or absence of a steric hindrance. BMPs with large bulky sidechains at this position such as GDF-5 of the pre-helix loop can only bind to BMPR-IB, whereas BMPs with small sidechains such as BMP-2 or BMP-4 can bind both BMP type I receptors equally well (Kotzsch *et al.*, 2009).

Analysis of this BMP-2 like GDF-5 variant revealed that in a cell line (ATDC5) having pro-chondrogenic properties and not expressing the BMPR-IB receptor this variant now has the same signaling properties and efficiency as BMP-2 (Nickel *et al.*, 2005). Thus under these conditions GDF-5 can signal via the BMPR-IA receptor and signaling efficiency is only decreased by the lower affinity of wildtype GDF-5 for BMPR-IA. Most interestingly, despite having the same receptor binding properties as BMP-2, GDF-5 R438A still does not induce ALP expression in the myoblastic cell line C2C12 (Klammert *et al.*, 2011). As RT-PCR analysis did not reveal significant differences in BMP receptor expression between both cell lines, ATDC5 and C2C12, other mechanism must exist that determine whether GDF-5 can fully signal through a particular BMP type I receptor. This observation also indicates that GDF-5 by binding to BMPR-IA can activate signaling on some cell types whereas on other cell types it might compete with BMP-2 for BMPR-IA and act as an antagonist (Klammert *et al.*, 2011). The mutation found in SYM1 affected humans, R438L, does not show a complete loss in BMP type I receptor specificity, the larger leucine sidechain in comparison to alanine leads to a 6 to 9-fold higher affinity to BMPR-IB compared to BMPR-IA (Seemann *et al.*, 2005, Kotzsch *et al.*, 2009). However, the result will likely be similar as above in that the mutation R438L renders GDF-5 into a protein that has BMP-2 like receptor binding properties. As BMP-2 is assumed to induce or at least regulate apoptosis in the interdigital mesenchyme (Yokouchi *et al.*, 1996, Merino *et al.*, 1999a), one would first expect increased apoptosis in patients carrying the mutation R438L in GDF-5 due to the presence of an additional BMP-2 like factor (Seemann *et al.*, 2005). However, our latest observation that increased BMPR-IA binding by GDF-5 R438A might not induce full signaling in all cell types possibly indicates that here the gain-of-function mutation in GDF-5 surprisingly leads to a loss of BMP-2 signaling in certain areas of the developing joint by competing for the binding to the same receptor BMPR-IA thereby might impede BMP-2 induced apoptosis which finally results in joint fusion (Klammert *et al.*, 2011).

4. Conclusion

When GDF-5 was discovered, due to its highly defined expression pattern during limb development, which precisely correlates with the location of all future joints throughout the limb, it was assumed immediately that this particular TGF-β factor takes the center stage in the development of all synovial joints. It thus came as a surprise when the *GDF5* knockout mice despite being affected in joint and limb development still showed multiple joints being developed quite normally. Genetic and functional analyses of human skeletal malformation diseases such as brachydactyly or chondroplasia showed that not only a number of other genes can lead to loss of joints or limb deformations similar to those seen in the *GDF5* null mice, but that also different mutations in GDF-5 can result in very distinct malformation phenotypes. Further studies revealed that often these different factors, many of them acting as morphogens themselves, such as Wnts and its (co-)receptors, members of the Sonic Hedgehog family or the FGFs, do not act independently but can be upstream or downstream of the TGF-β signaling cascade or even form positive or negative feedback loops with signaling components of the TGF-β superfamily. This complex regulatory network is further complicated by the fact that components of the TGF-β superfamily - ligands, receptors as well as antagonists – are known to function via highly promiscuous protein-protein interactions. Even if we restrict our focus onto the regulatory signaling network of GDF-5, its highly overlapping receptor binding specificities with other BMPs, such as BMP-2, BMP-6 or BMP-7, all of which are expressed in the direct neighborhood of the developing joint, make immediately clear that mutations altering binding of one particular ligand-receptor pair will ultimately affect the signaling output of other BMP members even when those are not affected by mutations themselves.

One mutation in GDF-5 – R438L – best exemplifies the dilemma. This mutation enables GDF-5 to now efficiently bind to a second BMP type I receptor, BMPR-IA. However this receptor is usually utilized by BMP-2 also present during joint development. As it is not known whether the GDF-5 variant with the altered type I receptor specificity delivers the same signal via this receptor as BMP-2 or whether it can signal at all through this BMP receptor in the present cellular context, developing a molecular disease mechanism explaining the mode of operation for this mutant seems impossible. In addition to this fuzzy BMP ligand-receptor network modulators like Noggin act like hub proteins interacting with multiple BMP ligands with a distinct BMP specificity profile. These interactions are again often linked to feedback loops leading to a precisely defined equilibrium of BMPs, BMP receptors and other modulators, which as a sum deliver a defined biological outcome. Classical morphogens such as the BMPs are considered to function via a concentration gradient, which is then interpreted by the different cells by responding to a particular morphogen threshold. However, the discrepancy of strong *GDF5* expression in all future joint locations and the highly localized effect seen in *GDF5* knockouts suggests that responsiveness to or the differentiation program run by GDF-5 is encoded along the digital ray by the various other morphogens in a temperospatial manner, thus allowing to run the differentiation program for joint formation by GDF-5 only at certain times at very defined places, whereas at other places or at earlier or later developmental stages as defined other factors will take over the GDF-5 function.

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