Expression and functional analysis of *EFNB1* mutations in craniofrontonasal syndrome

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1. Introduction

1.1. Ephrin binding receptors (Eph)

Ephrin binding receptors comprise the largest subfamily of receptor protein tyrosine kinases. This subfamily consists of 14 members and is divided into two groups based on the similarity of their extracellular domain sequences and their affinities for binding ephrin-A or ephrin-B ligands. According to the Eph Nomenclature Committee (1997) Eph receptors interacting preferentially with ephrin-A proteins are called EphA and Eph receptors interacting preferentially with ephrin-B proteins are called EphB. Mammals have nine A-subclass Eph-receptors (EphA1 to EphA9) and five B-subclass Eph-receptors (EphB1 to EphB5) (Yamaguchi and Pasquale, 2004). The Eph receptor family plays an important role in tissue development including neuronal and vascular networks during embryogenesis. The functions of Eph receptors in the adult brain have only recently been investigated. Y. Yamaguchi and E. Pasquale (2004) have shown that the Eph receptors regulate the structure and physiological function of excitatory synapses and might play a significant role in higher brain functions. It was shown that the Eph receptors and their ephrin ligands play a critical role in modulating multiple aspects of synaptic structure and physiology. Historically, these proteins have been best known for their role in axon guidance during the assembly of neural network (Wilkinson, 2001).

Binding of Eph receptors to their ligands induces receptor dimerization and subsequent *trans*-phosphorylation by the cytoplasmic kinase domains of the two receptors. Multiple tyrosine residues important for autophosphorylation and recruitment of adaptor proteins have been mapped to the juxtamembrane and kinase domains of the receptor.

To convert Eph receptors into the autoinhibited inactive state, their juxtamembrane tyrosine residues need to be dephosphorylated by a phosphotyrosine-specific protein phosphatase (PTP). Recent work identified protein-tyrosine phosphatase receptor type O (Ptpro) as a specific PTP that efficiently dephosphorylates EphA and EphB receptors (Shintani et al., 2006).

Many of the proteins identified in the Eph signalling pathways have been implicated in regulating cell morphology, attachment and motility. For example, Eph receptors are found to regulate integrin-dependent cell adhesion through activation of

c-Jun kinase via Nck-interacting Ste20 kinase in endothelial and neuronal cells (Becker et al., 2000; Huyn-Do et al., 1999). In addition, focal adhesion kinase (FAK) was implicated in the regulation of cell adhesion in prostate carcinoma PC-3 cells (Cheng et al., 2002). Eph receptor signalling also regulates actin dynamics via small GTPases of the Rho family. Taken together, these studies demonstrated the role of Eph receptor signalling in changing of cellular morphology and architecture, cell attachment and cell motility.

1.2. Ephrins

The ligands for Eph receptors have been named ephrins (Eph Nomenclature Committee, 1997). Based on their structures and sequence relationships, human ephrins are divided into the ephrin-A class, containing three proteins, which are anchored to the membrane by a glycosylphosphatidyl-inositol (GPI) linkage, and the ephrin-B class, containing five proteins, which are transmembrane proteins. Analyses of amino acid sequences of ephrin ligands indicate that each ligand consists of a signal peptide at the amino terminus, followed by a conserved receptor binding region containing conserved cysteine residues and a spacer region. At the carboxy terminus, the class A ligands contain a hydrophobic region comprising the GPI linkage. Ephrin-B ligands contain a transmembrane domain and a cytoplasmic domain containing PDZ-binding motif and conserved tyrosine residues that may be phosphorylated and serve as docking sites for proteins containing SH2/SH3 domains. Mostly ephrin-As bind to EphA receptors and ephrin-Bs bind to EphB receptors. But there are exceptions: most notable are ephrin-A5 that can bind to EphB2 receptor and ephrin-B1 that can bind to EphA4 receptor (reviewed in Kullander and Klein, 2004).

The cytoplasmic and transmembrane domains of ephrin-B ligands are analogous to those of conventional receptor molecules. Ephrin-B ligands share a single transmembrane domain, a cytoplasmic region, and a C-terminal PDZ binding motif. The cytoplasmic domains of ephrin-B ligands become phosphorylated on tyrosine residues following receptor binding (Brückner et al., 1998; Holland et al., 1996). PDZ domain-containing proteins, including Syntenin, Grip, Pick 1, Phip, the phosphotyrosine phosphatase FAP-1 were shown to interact with ephrin-B ligands (Lin et al., 1999; Torres et al., 1998), and PDZ-RGS, a PDZ containing protein with

GTPase stimulating activity, which regulates SDF-1 induced chemoattraction (Lu et al., 2001). Despite lacking a cytoplasmic domain, ephrin-A ligands also appear to be capable of signal transduction. There are precedents for GPI anchored proteins to signal, and a number of such proteins are involved in immune responses (Ropert and Gazzinelli, 2000). Recent data have shown that signalling through ephrin-A ligands may be mediated by the recruitment of adapter proteins. The ephrin-A ligands have been localized to lipid microdomains or rafts, which contain other signalling molecules such as G proteins and caveolin proteins, indicating ephrin-A ligands may activate a number of signalling pathways (Oh and Schnitzer, 2001). Stimulation of ephrin-A8 by EphA5–Fc has been shown to recruit and activate Fyn, a member of the Src kinase family. Fyn, in turn, activates p80 kinase to induce cytoskeletal changes in fibroblasts and increase cell adhesion to fibronectin through increasing the number of focal adhesions (Davy et al., 1999). Ephrin-A2 and ephrin-A5 have also been localized to lipid microdomains in HEK293 transfected cells. These observations indicate that ephrin-A ligands are capable of transducing signals to influence actin cytoskeletal changes, attachment, and migration.

Functional evidence of bi-directional signalling came from studies in zebrafish and homozygous null mice. Mellitzer et al. (1999) developed a zebrafish animal cap assay to study cell intermingling between two populations of cell expressing either Eph receptor or ephrin ligand. Juxtaposition of these two populations of cell leads to restriction of cell intermingling and establishment of boundary. However, expression of truncated forms of either EphB2 or ephrin-B2 lacking cytoplasmic domains in the animal cap results in cell intermingling across the border, indicating that signalling pathways directing cellular repulsion are activated through the ligand as well as the receptor. The functional role of ephrin cytoplasmic domain is demonstrated in ephrin-B2ΔC/ΔC mice expressing a mutant ephrin-B2 with cytoplasmic domain deletion (Adams et al., 2001). These mice exhibit vascular remodeling defects that are reminiscent of one subset of the phenotypes in ephrin-B2-/- null mice, indicating that the signalling through ephrin-B2 is required for vascular development. Interestingly, unlike ephrin-B2-/- null mice, no defects in neural crest cell migration are present in ephrin-B2ΔC/ΔC mice. These results indicate that ephrin-B2 signalling is required for vascular development, but is dispensable for neural crest cell migration during embryogenesis.

1.3. Eph–ephrin signalling system

In each subclass, A or B, receptor–ligand binding is promiscuous. With few exceptions, one ligand is able to bind to multiple receptors; and conversely, one receptor can bind to multiple ligands within the same subclass (Gale et al., 1996). Thus, the specificity of ligand–receptor interaction apparently comes from the cell-specific localization of these molecules *in vivo*. All other RTKs bind to soluble ligands, which can diffuse considerable distances, but ephrins require membrane attachment for proper functioning, limiting the action of this system to cell-to-cell communication. Another unique aspect of the ligand–receptor interaction in the Eph family is that the extent of receptor activation is dependent on the oligomerization state of the ligands. Soluble ephrin–Fc fusion proteins require preclustering (with anti-Fc antibodies) into aggregates to induce robust Eph phosphorylation and biological responses (Davis et al., 1994), whereas nonclustered forms can act as functional antagonists (Gerlai et al., 1999). The regulation of Eph–ephrin clustering under physiological conditions is not understood. Experiments with ephrin-A5-coated beads performed by Wimmer-Kleikamp et al (2004) suggested that ephrins induce Eph clustering, but that cluster growth occurs independently of ephrin contacts and involves direct Eph–Eph interactions.

Crystallographic studies indicate that binding of ephrins to Eph receptors is achieved by the formation of tetrameric structure comprising two receptor molecules and two ligand molecules (Himanen et al., 2001; Nikolov et al. 2005). The Eph–ephrin binding and the formation of tetrameric structure lead to conformational alterations in the B-class ephrin transmembrane and cytoplasmic domains. This could result in a more permissive conformation that allows phosphorylation of the ephrin cytoplasmic tail by adjacent Src family tyrosine kinases (SFKs) that are also localized in lipid rafts (Cowan C. and Henkemeyer M., 2002). The effects on receptor phosphorylation and activation of downstream signalling pathways differ when soluble recombinant ephrin ligands are presented to receptor either as ephrin–Fc dimers, or as clustered multimers. Both A and B class ephrins are capable of inducing signal transduction cascades upon binding Eph receptors. Following ephrin binding to its receptor, in addition to stimulating signalling cascades within the Eph-bearing cell (referred to as “forward signalling”), ephrins can elicit signals within the ephrin-bearing cell (“reverse” signalling). Both signalling events can happen

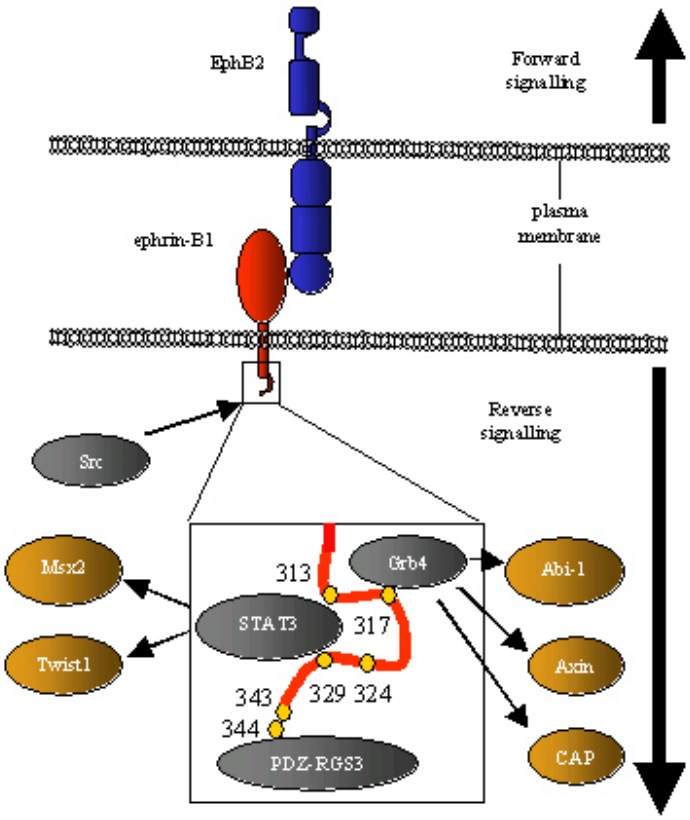
simultaneously, and the relative contributions of Eph forward and ephrin reverse signalling can vary depending on cellular context. Signalling events activated by Eph forward and ephrin reverse signalling are beginning to be characterized, and seem to be different from canonical RTK signalling that largely makes use of Ras–MAPK (mitogen-activated protein kinase) and phosphatidylinositol 3-kinase (PI3K)–Akt pathways (Eswarakumar et al. 2005). Ephs are poor activators of Ras–MAPK and PI3K–Akt pathways and, instead, recruit phosphotyrosine-binding adaptor proteins to activate Rho GTPases and remodel the actin cytoskeleton (Noren and Pasquale, 2004). Ephrin-B reverse signalling also activates Rho GTPases (involving Src family tyrosine kinases rather than using an intrinsic kinase domain) and uses phosphotyrosine-independent docking mechanisms (Cowan and Henkemeyer, 2001; Palmer et al., 2002).

Taken together, these findings suggest that Eph signalling requires a lot of factors in order to achieve precise *in vivo* regulation of the multiple functions, from embryonic patterning, neuronal targeting, to angiogenesis.

1.4. Ephrin-B1 and EphB2

EFNB1 and *EPHB2* are expressed throughout most tissues in vertebrate embryos. Together with the EphB2-receptor ephrin-B1 forms a signalling complex. Both ephrin-B1 and EphB2 can act as ligand and receptor (Schmucker and Zipursky, 2001; Cowan and Henkemeyer, 2002) as it was mentioned before. Therefore, EphB2–ephrin-B1 can act in a bi-directional signalling pathway (**Fig. 1**). Eph-receptors are activated when they are bound by clustered membrane-attached ephrin-ligands. Therefore, close contact between cells expressing Eph-receptors and ephrins is essential for receptor activation. By contrast, high-affinity interactions between ephrins and Ephs usually result in contact-mediated repulsion. Possible solutions to this paradox are ectodomain cleavage and endocytosis. Axon repulsion by ephrin-B1 requires cleavage of the ephrin-B1 ectodomain by γ-secretase in a presilin-dependent way (Tomita et al., 2006). This secretase was shown to associate with ephrin-B1 before cell–cell contact. Cleavage of the ephrin ectodomain by γ-secretase occurred *in cis* (with both proteins present in the same membrane) and proceeds independently from the ephrin-B1 – EphB complex formation. In it's turn, the C-terminus of ephrin-B1 regulates the exocytosis of matrix metalloprotease-8 (MMP-8),

a key protease of ephrin-B1 cleavage, in response to the interaction with EphB2 receptor (Tanaka et al., 2007). This group also showed that EphB2 – ephrin-B1 interaction promoted secretion of MMP-8 without increasing the expression level of MMP-8, proposing that ephrin-B1 signalling resulted in increased transport of MMP-8 from the cytoplasm to the cell surface (Tamaki and Yamashina, 2002).



Picture 1. The scheme of EphB2-ephrin-B1 interaction.

A mechanism that specifically removes Eph–ephrin complexes from the cell surface and enables the detachment of cells is rapid internalisation (trans-endocytosis). In cell culture assays, the interaction of cells expressing EphB receptors with cells expressing ephrin-B1 results in the rapid formation of intracellular vesicles containing EphB–ephrin-B complexes in both cell populations (Zimmer et al., 2003). The balance between reverse, forward or bi-directional endocytosis might depend largely on the cellular context, but is also regulated by the intracellular

domains of the proteins: when an EphB2-expressing cell contacted a cell expressing a C-terminally truncated version of ephrin-B1, endocytosis of the complex was favoured into the EphB2-expressing cell. When cells expressing C-terminally truncated versions of EphB2 and ephrin-B1 interacted, neither of the two proteins was endocytosed and cells adhered for prolonged periods of time. When neurons expressing ephrin-B1 were confronted with a cell expressing a C-terminally truncated version of EphB2 (and, thereby, incapable of eliciting EphB2 endocytosis), they displayed slower detachment than neurons confronted with a cell expressing full-length EphB2 (Zimmer et al., 2003). In endothelial cells, internalisation of receptor–ligand complexes and cell retraction depend on actin polymerisation and Rac activity within the Eph-expressing cells (Marston et al., 2003). Cowan et al. (2005) have implicated the Rac exchange factor Vav in EphB-mediated endocytosis. It was suggested that trans-endocytosis of ephrin-Bs depends on a clathrin-mediated pathway and proceeds mainly through the EphB-receptors internalisation (Parker et al., 2004).

Eph-ephrin binding autophosphorylates several tyrosine residues in the intracellular part of the receptors. EphB2-receptor, which has a strongest affinity to ephrin-B1, comprises a kinase domain and a juxtamembrane domain. After juxtamembrane tyrosine residues are phosphorylated, this domain is released from the interaction with the kinase domain, allowing the kinase domain to convert into its active state (reviewed in Kullander and Klein, 2002). Eph receptors transduce forward signals into the cell through activation of their intracellular catalytic tyrosine kinase domain.

In contrast, ephrins do not have an intracellular kinase domain and must therefore transduce their reverse signals in a different way. Reverse signalling may be enhanced by the ability of the Eph–ephrin tetramers to aggregate into higher-order clusters within the lipid rafts, which leads to the formation of discrete signalling centres. The cytoplasmic tail of ephrin-B1 contains several very conserved amino acids, including six phosphorylated tyrosines corresponding to Tyr313, Tyr317, Tyr324, Tyr329, Tyr343 and Tyr344 in human ephrin-B1 protein (**Fig. 1**). These tyrosines become phosphorylated after the engagement with the cognate EphB receptors, four of them (Tyr313-Tyr329) are the main targets of claudin4-iduced phosphorylation (Tanaka et al., 2005). Tyr313 is involved in STAT3 – ephrin-B1 interaction (Bong et al., 2007); Tyr317 forms a domain YEKV for Grb4 docking (Bong

et al., 2004; Su et al., 2004). Tyr324 was shown to be important for EphB2 – ephrin-B1 reverse signalling (Kalo et al., 2001), however, function of this tyrosine is still not clear. Tyr329 is involved in STAT3 – ephrin-B1 interaction (Bong et al., 2007) and Par-6 – ephrin-B1 binding (Lee et al., 2008). Tyr343 forms clathrin endocytosis signal motif – YXXφ (Bonifacino and Trub, 2003; Parker et al., 2004). Tyr344 is a part of PDZ-binding domain (YKV). Like cytokine receptor signalling, the phosphorylation of B-ephrin cytoplasmic tyrosines is thought to be important in the transduction of reverse signals (Cowan and Henkemeyer, 2002). It was shown by Brückner et al. (1997) that in fibroblasts ephrin-B1 phosphorylation is initiated by platelet-derived growth factor (PDGF) *in cis*. Palmer et al. (2002) identified Src family kinases (SFKs) and the PDZ domain-containing phosphatase PTP-BL as regulators of further ephrin-B1 phosphorylation and reverse signalling in fibroblasts. Tyrosinephosphorylated ephrin-B1 molecules recruit the adaptor protein Grb4 (growth-factor-receptor-bound protein 4), which consists of one SH2 and three SH3 domains and can increase FAK (focal adhesion kinase) catalytic activity as well as cell rounding (Cowan and Henkemeyer, 2001). Binding of Grb4 to ephrin-B1 requires 22 amino acids of the cytoplasmic region, which is identical between ephrin-B1 and ephrin-B2 and contains three of the six conserved tyrosine residues. An NMR binding study identified two tyrosine residues, corresponding to Tyr 313 and Tyr 329, within the 22-residue hairpin of ephrin-B1 as phosphorylation sites that may be relevant for high-affinity binding to the Grb4 SH2 domain (Song, 2003). However, the critical tyrosine residue involved in the binding of Grb4 SH2 domain is not yet clear. Bong et al., (2004) show that an FGFR can induce the phosphorylation of ephrin-B1 and promote the formation of a complex between ephrin-B1 and Grb4. This group also define the critical region within ephrin-B1 required for binding Grb4 and identify Tyr-298 (corresponding to 313 in human ephrin-B1) as vital for binding the Grb4 SH2 domain *in vivo*. They suggest that ephrin-B1 reverse signalling may be modulated by FGF (fibroblast growth factor) signalling and that the Grb4 adaptor protein may participate in this signalling as a result of being recruited to the phosphorylated Tyr 298 site.

Claudins are tetraspan transmembrane proteins comprising a multi-gene family with more than 20 members, and creating a complex with ZO-1, ZO-2 and ZO-3, which represent plaque structures underlying plasma membranes (Itoh et al, 1999; Tsukita et al, 2001). Although ephrin-B1 interacts with claudins on the same epithelial cell surface *in cis*, the tyrosine phosphorylation of the cytoplasmic region of ephrin-B1

was found to be markedly enhanced by the cell–cell contact formation in a manner dependent on claudin (Tanaka et al., 2005). This group also has shown that ephrin-B1 and claudin associate only *in cis*, in the same membrane, but not *in trans*, in opposing membranes. Par-6 is a major scaffolding protein that constitutively binds atypical protein kinase C (aPKC) through its PB1 (Phox and Bem 1) domain; binding stimulates a conformational change in an active Cdc42-GTP, causing aPKC activation in epithelial cells (Joberty et al., 2000). Lee et all (2008) present evidence that Par-6 has a crucial role in the ability of ephrin-B1 to regulate tight junction formation. Par-6 is a member of Par polarity complex, the localized activity of this complex (Par-6/aPKC/Cdc42-GTP) to the apical cell junctions regulates tight junction formation, and tight junction complexes are associated with the actin cytoskeleton, which is reorganized for the formation and maintenance of cell–cell contacts (Lee et al., 2008). Ephrin-B1 competes with Cdc42 for association with Par-6. This competition leads to the inactivation of Par complex and loss of tight junction. Interaction between ephrin-B1 and Par-6 is disrupted by tyrosine phosphorylation of the intracellular domain of ephrin-B1. Lee et al. (2008) expect this to be a mechanism by which ephrin-B1 signalling regulates cell–cell junctions in epithelial cells. They also showed that the most crucial tyrosine in Par-6 – ephrin-B1 interaction corresponds to Tyr 329 in human ephrin-B1 protein.

Palmer et al. (2002) showed that in primary endothelial cells SFKs are activated upon binding to ephrin-B1 ligands and are required for ephrin-B1 tyrosine phosphorylation and ephrin-B1-mediated angiogenic sprouting of the cells, however, SFKs are not required for all angiogenic stimuli. The responses of endothelial cells to other potent angiogenic factors such as the chemokine SDF-1, via its receptor CXCR4, and Ang-1, via its receptor Tie-2, were not affected by inhibition of SFKs (Palmer et al., 2002). Although SFKs are rapidly and stably recruited to ephrin-B1-containing membrane clusters, their activation is transient.

In PC-3 cells, ephrin-B1 ligands recruit PTP-BL to the membrane via PDZ domain interactions and become dephosphorylated. PTP-BL contains five PDZ domains, which interact with other cytoplasmic proteins including a GTPase-activating protein (GAP) with specificity for the Ras-like GTPase Rho (Saras et al., 1997).

Chong et al., (2000) demonstrate that FGF receptor activation inhibits x-ephrin B1-induced cell dissociation in *Xenopus* embryos. They show that the fibroblast growth factor (FGF) receptor associates with and induces the phosphorylation of x-ephrin-

B1. This group also define critical residues within a conserved region of the intracellular domain necessary for this interaction. These results prove a functional and physical interaction between the FGF tyrosine kinase family of receptors and the ephrin-B1 ligand and implicate their cross talk in regulating adhesion.

Ephrin-B1 protein is located in lipid rafts (Brückner et al., 1999), however, raft disruption by cholesterol depletion in NIH3T3-sites in ephrin-B1 cells does not affect the clustering of ephrin-B1 induced by the receptor and the subsequent recruitment of Src into these clusters (Palmer et al., 2002). Lipid rafts are small (<100 nm) membrane domains (Friedrichson and Kurzchalia, 1998; Varma and Mayor, 1998), rich in sphingolipids and cholesterol. Lipid rafts can incorporate GPI-anchored proteins, specific transmembrane proteins, and doubly acylated proteins like tyrosine kinases of the Src family (Simons and Ikonen, 1997). Rafts function as platforms for the assembly of cytoplasmic and membranous signalling molecules and for polarized membrane traffic (Holowka and Baird, 1996; Simons and Ikonen, 1997). In the signalling of membrane receptors, adaptor proteins provide docking sites for downstream enzymes in proximity to the activated receptor. In HEK 293 cells Brückner et al. (1999) describe the interaction of ephrin-B1 with the multi-PDZ domain protein glutamate receptor–interacting protein (GRIP), a protein, termed GRIP2 that contains seven PDZ domains (PDZ1-7). Ephrin-B1 is associated with sphingolipid/cholesterol-rich raft membrane microdomains and recruits a significant fraction of GRIP proteins from the cytoplasm into these rafts. Stimulation with EphB2 receptors reorganizes ephrin-B1-containing rafts into larger raft patches that concentrate GRIP proteins on the cell surface.

To transduce reverse signals, ephrin-B1 cytoplasmic tails associate with other cytoplasmic proteins. They appear to be proteins containing PDZ (postsynaptic density-95, disclarge and zonulin-1 proteins) domains, such as PDZ-RGS3 protein, which have been shown to bind to the carboxyl termini of the ephrins of B-class (YKV). At least one protein containing a Src homology 2 (SH2) domain, Grb4, is recruited to these ephrins cytoplasmic tails in a phosphotyrosine-dependent manner. Both types of ephrin-interacting protein have been reported to direct signals that regulate cytoskeletal dynamics (Cowan and Henkemeyer, 2002). In neurons, ephrin-B1 reverse signals promote the maturation of spines through a signalling pathway involving Grb4, G protein-coupled receptor kinase-interacting protein (GIT1) and the exchange factor for Rac GTPase, β-PIX (Segura et al., 2007). The activation of Src

kinases involves metalloproteinase and presenilin-1–γ-secretase cleavage of ephrin-B1, producing an intracellular peptide that binds to and activates Src by inhibiting its association with the inhibitory kinase Csk (Tomita et al., 2006). This process occurs independently from the ligand-receptor binding.

The signalling pathway downstream of ephrin-B1 involves Dishevelled (Dsh), which binds either directly to the cytoplasmic tail of ephrin-B1, or indirectly through interaction with Grb4 (Tanaka et al., 2003). Dishevelled (Dsh) is a cytoplasmic protein containing three conserved protein domains: DIX (Dishevelled-Axin), PDZ and DEP. Dsh is known to be located as a downstream molecule of Frizzled (Frz) and involved in at least two independent signalling pathways in *Drosophila*, *Xenopus* and zebrafish. One pathway is a canonical cascade that leads to the dorsalization and determination of the body axis by interacting with β-catenin and the other is a planar cell polarity (PCP) cascade (Tanaka et al., 2003).

Previously, it was reported by Bong et al. (2007) that STAT3 is important for ephrin-B1 signalling. Among the six tyrosine residues present in the intracellular domain of ephrin-B1 (see above), one resides in a well conserved YXXQ motif at the C-terminus and is rapidly phosphorylated upon engagement of the EphB ectodomain (Kalo et al., 2001) or in response to FGF receptor activation (Cong et al., 2000, Bong et al., 2004). STAT3 plays pivotal roles in development, proliferation, apoptosis and tumorigenesis. STAT3 is activated in response to several agents, including growth factors, cytokines, and oncoproteins (Bong et al., 2007). The activated STAT3, dimerizes, translocates to the nucleus, recognizes target DNA, and induces gene expression (Taga and Kishimoto, 1997).

Twist1, a highly conserved basic helix-loop-helix transcriptional factor, was previously shown to play a pivotal role in mesodermal, myoblast, and osteoblast differentiation (Bialek et al., 2004; Baylies and Bate, 1996; Castanon et al., 2001). Mutational inactivation of the *Twist1* gene resulted in Saethre-Chotzen syndrome (OMIM 101400), an autosomal dominant disorder characterized by premature fusion of the cranial sutures, skull deformations, limb abnormalities, and facial dysmorphisims (El Ghouzzi et al., 1997, Howard et al., 1997). Cheng et al. (2008) detected an elevated level of activated STAT3 in the highly invasive colon carcinoma cells and demonstrated its role in transcriptional regulation of Twist1. This group showed that STAT3 bound to the *Twist1* promoter and activated its transcriptional activity. They also demonstrate that *TWIST1* is a transcriptional target of STAT3.

Contractor et al. (2002) show that dimeric EphB-Fc fusion proteins can directly activate presynaptic ephrin-B1 ligands and potentiate mossy fiber synaptic transmission, thus occluding subsequent tetanus-induced LTP (long terminus potential) in neurons.

In summary, ephrin-B1 and EphB2 are involved in processes of cell adhesion and cell migration (Böhme et al., 1996). Therefore, this signalling system plays an important role in embryonic morphogenesis including definition of tissue borders and segmentation. So far, analysis of Eph/ephrin system focused on its significance for skeletal development, angiogenesis and plasticity of the nervous system. Ephrin-B1 plays an important role for axon guidance and plasticity of neurons. Ephrins form a high-affinity multivalent complex with their receptors present on axons. After Eph/ephrin engagement axons can be rapidly repelled (Murai and Pasquale, 2003). Furthermore, ephrin-B1 is involved in other processes such as cancerogenesis.

1.5. Ephrin – Eph signalling in vascular and nervous systems

Lim et al. (2008) examined the regulatory function of ephrin-B reverse signalling on synapse development soon after the establishment of retinotectal projections in the *Xenopus* tadpole. Using *in vivo* timelapsed imaging of developing RGC axons in the tectum, they found that acute elevation of ephrin-B signalling accelerates presynaptic maturation without affecting the overall axonal growth. Functional synaptic maturation was facilitated by ephrin-B signalling in two apparent phases: an early phase of presynaptic enhancement of transmitter release and a late phase of postsynaptic increase in the glutamate responsiveness, both of which contribute to an elevated AMPAR-mediated synaptic transmission.

EphB1 is largely confined to the nervous system, and ephrin-B3 and EphB2 are expressed in neuroectoderm and heart (Henkemeyer et al. 1996). Previous studies on *Efnb3*$^{-/-}$ gene deletion (Rodenas-Ruano et al., 2006) and hippocampusspecific *Efnb2*$^{-/-}$ conditional deletion (Grunwald et al., 2004) in mice showed no structural alteration in synaptic morphology in the hippocampus, although the level of synaptic proteins was altered in Efnb3$^{-/-}$ mice (Rodenas-Ruano et al., 2006).

Eph–ephrin signalling has also been implicated in trans-endocytosis events at the neuron-to-glia interface. Using time-lapse microscopy, it was shown that hippocampal neurons exogenously expressing EphB2 receptors release or pinch-off EphB2-

containing vesicles from the neuron at sites of neuron-to-glia contact. These findings suggest that Eph–ephrin signalling is involved in the glial cell-mediated fine sculpting of neuronal structures (Lauterbach and Klein, 2006).

EphB forward signalling might not always be linked to EphB endocytosis, but might modulate the endocytosis of other transmembrane proteins. In cultured hippocampal neurons, EphB receptors regulate clathrin-mediated endocytosis of α-amino-3-hydroxy-5-methyl-4-isoxazolpropionic acid (AMPA)-type glutamate receptors, a mechanism implicated in long-term synaptic plasticity (Irie et al., 2005). These authors identified synaptojanin-1, a phosphatidylinositol 5'-phosphatase that is involved in clathrin-mediated endocytosis, as a physiological substrate for EphB2. Because synaptojanin-1 tyrosine phosphorylation was crucial for its role in AMPA receptor endocytosis, this work highlighted the importance of tyrosine kinase signalling in this process.

As cell surface bound molecules, Eph and ephrin signalling mediates cell-to-cell contacts. During neural development, Eph receptors and ligands often display reciprocal expression patterns on adjacent tissue compartments such as between neighbouring rhombomere segments or between projecting axons and their targets. Bi-directional signalling of Eph receptors and ephrin ligands mediates cell–cell repulsion to establish rhombomere boundaries and to guide axons to their targets (Flanagan and Vanderhaeghen P., 1998; Klein, 2001). In addition to mediating cellular repulsive signals, interaction of two cell populations expressing either EphB receptors or ephrin-B ligand reduces the number of gap junctions between these two populations of cells, preventing the communication of these two groups of cells (Mellizer et al., 1999).

The family of Eph receptor tyrosine kinases and their ephrin ligands play important roles in a variety of processes during embryonic development of mammals, lower vertebrates, and invertebrates such as *Caenorhabditis elegans* (Adams et al., 1999). They regulate topographic map formation in the retinotectal/retinocollicular system (Cheng et al. 1995; Drescher et al. 1995; Nakamoto et al. 1996; Frisen et al. 1998) and play essential roles in the formation and fasciculation of brain commissures (Henkemeyer et al. 1996; Orioli et al. 1996; Park et al. 1997). They have additional important functions in patterning of embryonic structures of the brain (Xu et al. 1995) and somites (Durbin et al. 1998). Ephrins control migration of neural crest cells into branchial arches and somites (Krull et al. 1997; Smith et al. 1997; Wang and

Anderson 1997). In these systems, ephrin–Eph interactions are thought to be mainly repulsive, that is, navigating growth cones or migrating cells expressing Eph receptors would turn away from cells expressing the corresponding ephrin ligand (Drescher et al. 1995; Nakamoto et al. 1996; Brennan et al. 1997; Krull et al. 1997; Wang and Anderson 1997). Repulsive interactions and complementary expression patterns suggest that ephrins and Eph receptors define spatial boundaries in the developing embryo (Gale et al. 1996).

It is known that Eph receptor tyrosine kinases and their cell-surface-bound ligands, the ephrins, regulate axon guidance and bundling in the developing brain, control cell migration and adhesion, and help patterning the embryo (Adams et al., 1999). This working group also reported that two ephrin-B ligands (ephrin-B1 and ephrin-B2) and three EphB receptors (EphB2, EphB3 and EphB4) are expressed in and regulate the formation of the vascular network. Mice lacking ephrin-B2 and double mutants deficient in EphB2 and EphB3 receptor signalling die *in utero* because of defects in the remodelling of the embryonic vascular system. Interaction between ephrin-B2 on arteries and its EphB receptors on veins suggests a role in defining boundaries between arterial and venous domains. Expression of ephrin-B1 by arterial and venous endothelial cells and EphB3 by veins and some arteries indicates that endothelial cell-to-cell interactions between ephrins and Eph receptors are not restricted to the border between arteries and veins. Furthermore, expression of ephrin-B2 and EphB2 in mesenchyme adjacent to vessels and vascular defects in ephB2/ephB3 double mutants indicate a requirement for ephrin–Eph signalling between endothelial cells and surrounding mesenchymal cells. Finally, ephrin-B ligands induce capillary sprouting *in vitro* with a similar efficiency as angiopoietin-1 (Ang1) and vascular endothelial growth factor (VEGF), demonstrating a stimulatory role of ephrins in the remodelling of the developing vascular system (Adams et al., 1999).

The assembly of endothelial cells and supporting mesenchyme into mature and functional blood vessels is a multi-step process involving a diverse array of molecular signals. Ligands for receptor tyrosine kinases (RTK) such VEGFs and angiopoietins are well known as critical mediators of blood vessel formation during embryogenesis.

Expression of ephrin ligands and Eph receptors during embryogenesis is highly dynamic, with expressions in various sites during different stages of development (Holder and Klein, 1999). These sites include the organizer, or node, during

gastrulation, different rhombomere segments during hindbrain patterning, neural crest cells that migrate to branchial arches during cranial morphogenesis, somites, and developing vasculature. Recently, class B members of the Eph/ephrin family have also been described in the developing cardiovascular system. Most interestingly, expression of *LacZ* reporter gene under the control of endogenous ephrin-B2 or EphB4 promoter/enhancer in mice lacking one allele of ephrin-B2 or EphB4 revealed complementary expression of ephrin-B2 in arterial endothelial cells and its cognate receptor, EphB4 in venous endothelial cells (Wang et al., 1998). Although later studies also found low levels of EphB4 on arterial endothelial cells and ephrin-B2 on paravascular supporting cells (Adams et al., 1999), ephrin-B2 and EphB4 are the best markers available for arterial and venous endothelial cells at very early stage of development. Other EphB molecules were also expressed at the sites of neovascularization. Ephrin-B1 is co-expressed with EphB1 in endothelium in the developing kidney (Daniel et al., 1996). EphB3 is expressed predominantly in veins, as is EphB4, but is also detected in some arteries such as those found in the yolk sac (Adams et al., 1999). In addition to expression in endothelial cells, ephrin-B2 and EphB2 are also expressed in mesenchymal supporting cells, suggesting a role of Eph receptors and ephrin ligands in vessel wall development through interactions between endothelium and mesenchyme (Adams et al., 1999).

1.6. *EFNB1* gene and CFNS

Human *EFNB1* (OMIM 300035) gene is located on the X-chromosome (Xq12-Xq13.1) in contrast to *EFNB2* and *EFNB3* genes that are located on the 13th (13q33.3) and 17th (17p13.1) chromosomes respectively. The genomic length of *EFNB1* is 13,17 Kb including the 8,7 Kb first intron, coding part consists of 1041 bp and encodes a polypeptide of 346 amino acids. *EFNB1* consists of 5 exons and 4 introns. Exons 2 and 3 (278 bp and 93 bp, respectively) encode the extracellular ephrin domain, exon 5 is 413 bp long and encodes transmembrane and intracellular domains. Exon 1 is 128 bp long and encodes the signalling peptide and exon 4 is 129 bp long and encodes a protein link between extracellular domain and the transmembrane part.

Most *EFNB1* mutations responsible for CFNS constitute point mutations, small deletions, insertions or duplications (Shotelersuk et al., 2006; Torii et al, 2007; Twigg

et al., 2004; Twigg et al., 2006; Vasudevan et al., 2006; Wieland et al., 2004; Wieland et al., 2005). Mutations of *EFNB1* were detected in CFNS patients in both familial and sporadic cases. Partial or complete *EFNB1* gene deletions were described in only several CFNS cases (Twigg et al., 2006; Wieland et al., 2004; Wieland et al., 2007). The major types of mutations (up to 55%) are frameshift, nonsense, and splice site mutations that lead to premature termination codons (PTCs). Missense mutations constitute about 42% of all *EFNB1* mutations (Twigg et al. 2004; Wieland et al. 2005). Most of them occur in exons 1-4 (ephrin extracellular domain), leading to the exchange of amino acid residues that are important for receptor–ligand interaction and bi-directional signalling. Nonsense mutations were found mostly in exons 1-3 (Wieacker and Wieland, 2005; Twigg et al., 2006). Frameshifts were detected in all 5 exons of *EFNB1*. Loss of gene function has been proposed for most mutations and has been shown for some of them, but it has not been proven for missense mutations, splice site mutations, and for mutations causing premature termination in exons 4 and 5 (Shotelersuk et al. 2006; Twigg et al. 2006; Wallis et al. 2008; Wieland et al. 2004, 2007, 2008).

Twigg et al. (2006) showed that ≈92 % of all *de novo* germline *EFNB1* mutations had a paternal origin. Also this working group reported that these mutations appeared in the male germline independently from the paternal age. The major part of *EFNB1* mutations occurs *de novo*. Wieland et al. (2005) described 26 novel mutations, sporadic cases consist 78,8 % of them, Twigg et al. (2006) described 39 new *EFNB1* mutations, sporadic cases consist 61,5 % of them. Some mutations such as c.196C>T/R66X and c.161C>T/p.P54L were detected both in family and sporadic patients (Twigg et al., 2006, Wieland et al., 2004; Wieland 2005). It was calculated that 80% of individuals harbouring *EFNB1* mutations would be female, which accords with clinical observations (Twigg et al., 2006).

Alternative splicing of the *EFNB1* gene is caused by splice-site mutations including the c.406+2T>C mutation that is described in this work. No alternative splice-site variants were described in normal situation till now.

More than 100 SNPs have been described for EFNB1 gene (NCBI SNP database http://www.ncbi.nlm.nih.gov/SNP/).

It was proposed by John et al. (2004) that expression of *EFNB1* is controlled by miRNA. This working group has identified miRNAs that can target ephrin-encoding genes (five genes including *EFNB1* out of seven genes). Although, many members of

the ephrin family are predicted targets of brain-expressed miRNAs, they appear to be targeted by different miRNAs, consistent with differential regulation (John et al., 2004).

MicroRNAs (miRNAs) interact with target mRNAs at specific sites to induce cleavage of the message or inhibit translation. Several hundred genes encoding transcripts containing short double-stranded RNA hairpins, named microRNAs (miRNAs), are identified in plants and animals up to now (Lee et al. 1993; Reinhart et al., 2002; Lagos-Quintana et al. 2001, 2002, 2003; Lau et al. 2001; Lee and Ambros 2001; Mourelatos et al. 2002; Ambros et al. 2003; Aravin et al. 2003; Brennecke et al. 2003; Dostie et al. 2003; Grad et al. 2003; Houbaviy et al. 2003; Lai et al. 2003; Lim et al. 2003a, 2003b). The cellular functions of most animal miRNAs are unknown. In eukaryotes miRNA genes constitute about 1%–2% of the known genes.

MicroRNAs working mechanism has specificity. In living organisms, miRNAs are differentially expressed in developmental stages, cell types, and tissues (Lee and Ambros 2001; Lagos-Quintana et al. 2002; Sempere et al. 2004). In particular, differential expression has been observed in mammalian organs (Lagos- Quintana et al. 2002; Krichevsky et al. 2003; Sempere et al. 2004) and embryonic stem cells (Houbaviy et al. 2003).

MicroRNAs cause the translational repression or cleavage of target messages (Doench and Sharp 2004). Some miRNAs work like small interfering RNAs (siRNAs). They direct mRNA cleavage between the nucleotide positions 10 and 11 in the siRNA:mRNA target duplex (Tuschl et al. 1999; Zamore et al. 2000; Elbashir et al. 2001; Hutvágner and Zamore 2002a; Martinez et al. 2002; Bartel 2004; Yekta et al. 2004). Extent of base pairing between the small RNA and the mRNA determines the balance between cleavage and degradation (Hutvágner and Zamore 2002b). Cleavage of mRNA is a straightforward process, but the details of the mechanism of translational repression are still unknown. The rules of regulation in the siRNA pathway also apply to miRNA target recognition (Hutvágner and Zamore 2002b; Doench et al. 2003).

It was reported recently that Notch signalling activates the expression of *EFNB1* gene in mouse intestinal epithelium (Koo et al., 2009).

More than 100 SNPs have been described for *EFNB1* gene (NCBI SNP database, http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp&cmd). But none was detected in any patient group.

Craniosynostosis is a common birth defect (1 in 2100–2500 infants) that affects the premature fusion of one or more of the cranial sutures and leads to an abnormal head shape (Feldman et al., 1997). Craniofrontonasal syndrome (CFNS; OMIM 304110) is an X-linked craniofacial disorder and belongs to the heterogeneous group of craniofrontonasal dysplasia (CFND). It is characterized by craniofacial asymmetry, hypertelorism, coronal synostosis, strabismus, bifid nasal tip, grooved nails, abnormalities of the thoracic skeleton as well as wiry and curly hair. Polydactyly and syndactyly may also occur. Rare manifestations are anterior cranium bifidum, axillary pterygium, joint abnormalities, cleft lip and palate, unilateral breast hypoplasia, asymmetric lower-limb shortness, corpus callosum hypoplasia and aplasia, umbilical hernia, and diaphragmatic hernia (reviewed by Wieacker and Wieland, 2005).

CFNS has a very unusual manifestation pattern, in which affected females show multiple skeletal malformations whereas only mild abnormalities such as hypertelorism are observed in male carriers (Feldman et al., 1997; Morris et al., 1987).

CFNS locus was located at Xp22 by Feldman et al. (1997). Linkage analysis performed by Wieland et al. (2002) revealed that a CFNS locus is located in the pericentromeric region of the X chromosome. The *EFNB1* gene was mapped in Xq12-Xq13.1 that is included in the CFNS interval (Wieland et al., 2002, 2004).

Compagni et al. (2003) reported that targeted inactivation of *Efnb1* (the mouse homologue of *EFNB1*) in mice caused high perinatal lethality, abdominal wall closure defects, and skeletal abnormalities, especially of the thoracic cage. The phenotype is more severe in female heterozygotes than in hemizygous males, and some defects, such as preaxial polydactyly, were detected only in heterozygotes. The abnormalities of development in these mice looked like the symptoms of CFNS in human. Interestingly, it was shown, that homozygous female mice with both mutant alleles of *Efnb1* have less severe manifestation than heterozygous females (Davy et al., 2006).

Together the localization of the CFNS-locus (Wieland et al., 2002) and the result of *Efnb1* knockout mice (Compagni et al., 2003; Davy et al., 2004) made *EFNB1* a strong candidate gene for causing CFNS. Wieland et al. (2004) demonstrated that *EFNB1* mutations cause CFNS. Mutations in *EFNB1* were detected in the vast majority of patients with familial and sporadic craniofrontonasal syndrome (Wieland et al., 2005; Twigg et al., 2004).

1.7. Hypothesis of cellular interference

As it was said before, CFNS is caused by *EFNB1* mutations, and this gene is located on the X-chromosome. Surprisingly, female carriers with one mutant and one wild type *EFNB1* alleles are affected more severely than male carriers, although male carriers do have neither wild type allele nor a homologue of the gene on the Y chromosome. How does it happen?

Johnson (1980) proposed the hypothesis of metabolic interference to explain the situation. This hypothesis is suitable for the cases, when two allelic genes encode different subunits of a multisubunit enzyme or structural protein.

Young and Moore (1984) proposed that CFNS caused male lethality.

Ephrins are expressed in a complex and temporally dynamic pattern during embryogenesis. Wieland et al. (2004) assumed that, in heterozygous females, ephrin-B1–expressing and ephrin-B1–deficient cells lead to disturbance of cell sorting and migration. This group also proposed the “cellular interference” hypothesis. The core of this hypothesis is following. Somatic human cells have forty-six chromosomes as a diploid set including two sex chromosomes (XY in males and XX in females).

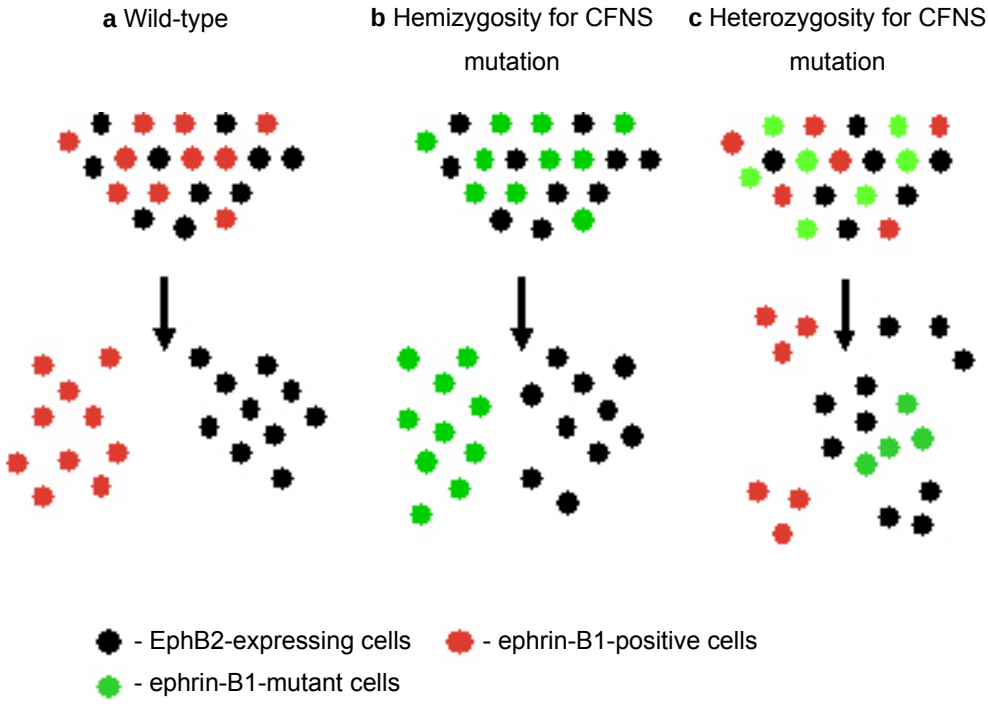


Fig. 2 Hypothesis of cell sorting in case of: **a** normal sorting; **b** male patient; **c** female patient.

Only one X-chromosome is active in a somatic cell – the single one in male cells and one of two in the female cells. In females the other X-chromosome is inactivated (Lyon, 1961) and subsists as a Barr body. This inactivation proceeds randomly in human. All females, including CFNS-patients, are functional mosaics because of this random X-inactivation. They have two different cell populations with different active X-chromosome. In case of female CFNS patients, cells of one population have the wild type *EFNB1* on the active X-chromosome and cells of the other population have the mutant type on the active X-chromosome. CFNS is caused by different sorting of the cells depending on the expression of the wild type or the mutant type *EFNB1* alleles (**Fig. 2c**). This sorting proceeds depending on interaction of ephrin-B1 with EphB-receptors. In males, this interaction proceeds normally (wild type *EFNB1*, **Fig. 2a**) or disturbed completely (mutant *EFNB1*) and, probably, rescued by some other ephrins (**Fig. 2b**). In females interaction proceed normally (wild type *EFNB1*, **Fig. 2a**) or disturbed partly due to random X-inactivation (mutant *EFNB1*) and, probably, partly rescued by some other ephrins (**Fig. 2c**). The interaction of EphB2-expressing cells with two populations of ephrin-B1-expressing cells (wild-type and mutant-type ephrin) is thought to be the major pathogenetic mechanism of this disease. The concept of cellular interference appears to be not unique for CFNS. Dibbens et al. (2008) described the molecular cause of epilepsy and mental retardation limited to females (EFMR, OMIM 300088). This X-linked disorder affects females, while male carriers are unaffected. EFMR is caused by mutations in *PCDH19* gene encoding the cell-cell adhesion molecule protocadherin 19. Like in CFNS, somatic mosaicism may cause cellular interference leading to malformations in the brain and development of epilepsy (Lindhout 2008). This pathogenic mechanism has been strongly supported by a mosaic male patient harbouring a *PCDH19* mutation, who was identified in a cohort of patients with Dravet syndrome-like epileptic encephalopathy (Depienne et al. 2009).

1.8. The aims of the work

The main aim of this work is expressional and functional analysis of different *EFNB1* mutations. Several patient derived mutations have been analysed. The work can be divided into two parts. First premature termination codon (PTC)-causing mutations were analysed: frameshift mutation c.377_384delTCAAGAAG (PTC in

exons 2/3 junction), frameshift mutation c.614_615delCT (PTC in exon 4) and splice-site mutation c.406+2T>C (PTC in intron 2 or exon 3). All three mutations were predicted *in silico* to result in a truncated, soluble ephrin-B1 protein.

In the second part of this work the impact of missense mutations c.161C>T/p.P54L and c.332C>T/p.T111I on cell behaviour and reverse ephrin-B1 cell signalling was investigated. The role of these mutations was analysed in a cell culture model using NIH3T3 fibroblasts. To analyse the impact of missense mutations in ephrin-B1 signalling the phosphorylation of t Tyr324 and Tyr329 amino acids in mutant and wild type ephrin-B1 was monitored after the EphB2-Fc stimulation.

As it was told already, ephrin-B1 is expected to be involved in STAT3, TWIST1 and MSX2 signalling pathways. According to that, the change of expression level of *MSX2* and *TWIST1* due to ephrin-B1 stimulation was monitored.

2. Materials and methods

2.1. Cells cultures work

2.1.1. Cell cultures maintaining

Informed consent was obtained from the parents of the patients. Fibroblasts of CFNS patients were obtained from skin biopsies (c.377_384delTCAAGAAG and c.406+2T>C mutations) or surgical therapeutic interventions (c.614_615delCT) into the synostosis. Primary cell cultures of patient fibroblast cultures were maintained according to standard cell culture conditions in tissue culture flasks (Cellstar®) with Dulbecco's modified Eagle's medium (DMEM, Sigma) containing 15 % fetal calf serum (FCS, Sigma) in a 5% CO_2 atmosphere at 37^{o}C. Every 4 days the exhausted medium was aspired and new medium added. Cells were harvested for genomic DNA and total RNA or protein isolation using Cell Scrapers (NUNC).

NIH 3T3 cells were cultivated in tissue culture flasks (Cellstar®) with DMEM containing 15 % FCS, in a 5% CO_2 atmosphere at 37^{o}C. Every 4 days the exhausted medium was aspired and new medium added. Cells were harvested for genomic DNA and total RNA or protein isolation using Cell Scrapers.

2.1.2. NIH 3T3 transfection

NIH 3T3 cells were placed on a 6-well plate (Greiner Labortechnik) at a ratio 1×10^5 cells/well, cells were counted using the cell counting camera (Neubauer). Cells were cultivated until they reached 70% confluence (usually within 24 h). Plated cells were cotransfected with pcDNA 3.1(+) vector (Invitrogen) containing the mutant or the wild type *EFNB1* cDNA (4 µg/well) and the pEGFP-N3 vector (4 µg/well, BD Biosciences). Transfections were done using PerFectin^{TM} Transfection reagent (PeqLab) according supplied protocol with modifications:

1. The cells were plated one day before transfection, so that they would be 70% confluent on the day of transfection.
2. On the day of transfection the hydrated PerFectin reagent was diluted with serum-free medium at a ratio: 28 µl PerFectin + 172 µl of serum-free medium per transfection reaction.

3. DNA was diluted with the DNA diluent at a ratio: 8 µg DNA + 200 µl diluent per transfection reaction. The solution was mixed well by pipetting several times and incubated for 5 min at room temperature.
4. After the 5 min incubation the DNA solution was added to the diluted PerFectin reagent. The mix was incubate at room temperature for 30 min to form PerFectin /DNA complexes (lipoplexes). After the incubation serum-free medium was added to the complexes to make up the transfection volume up to 1 ml per reaction.
5. The culture medium was aspirated from cells and cells were washed with PBS (Sigma).
6. Solution from the p. 6 was added to the cells (1 ml per well). Cells were incubated for 4 hours at 37^{o}C and 5% CO_2.
7. One volume of the medium containing 20% serum (1 ml per well) was added to the transfection reactions.
8. Fresh growth media was added 24 hours post transfection (1 ml per well).
9. The reporter gene assay was performed 24 to 72 hours following the transfection.

Transfection efficiency was measured using fluorescent cytometry cell sorting (FACS) 24 h post transfection as described below.

2.1.3. FACS analysis

Transfected cells were washed in PBS and treated with trypsin (Sigma) 24 h post-transfection. After the treatment, PBS was added and part of the cells was taken as a sample for the FACS analysis. FACS was performed using the ectopic GFP-fluorescence in BD FACSCanto^{TM} Flow Cytometer (BD Bioscience), 3×10^4 events were counted. Untransfected NIH 3T3 cells were used as a control.

2.1.4. NIH 3T3 stimulation

Stimulation of NIH3T3 cells with EphB2-receptor was performed according to a modified protocol of Davy et al. (2006):

1. The medium was aspired 32 h posttransfection, the cells were washed twice with PBS, the remaining liquid was drained off and cells starved 16 h in medium, containing 0,5 % serum.

2. EphB2-Fc/Fc (R&D Systems) was preclustered with anti-human rabbit IgG (R&D Systems) at a ratio 1:2 for 30 min at room temperature (EphB2-Fc/Fc was diluted to concentration 50 µg/ml with the serum-free medium). Preclustered EphB2-Fc/Fc was diluted to 4 µg/ml with the serum-free medium before adding to the cells. Preclusterization was performed directly before the stimulation
3. The low-serum medium was aspired, cells were washed twice with PBS, the remaining liquid was drained off and preclustered EphB2-Fc/Fc containing medium was added.
4. Cells were incubated for 5 to 30 min at 37^{o}C, washed in PBS and cultivated in DMEM with 15 % FCS as described above.

As a control, stimulation with preclustered Fc (R&D Systems) was performed.

2.1.5. Patches formation analysis

NIH 3T3 cells were transfected as it is described above, pictures of the transfected cells were taken 32 h posttransfection, just before the stimulation. Transfected NIH 3T3 were stimulated as it is described above. Stimulation lasts for 30 minutes. Patches formation analysis was done 24 h after the stimulation by taking photos. Pictures were taken by fluorescent microscopy method using Axiovert 25 Inverse Microscope and AxioCam MRc5 0450-354, Carl Zeiss.

2.2. Molecular methods

2.2.1. DNA extraction from fibroblasts

Extraction of DNA from fibroblasts was performed as it is described in Maniatis (1989) with changes:

1. The cells were washed twice with PBS (1ml/25cm^2), every time cells were scraped with a Cell Scraper into a microcentrifuge tube (Eppendorf).
2. The tubes with DNA were centrifuged for 5 min at 2500 min^{-1} at 4^{o}C
3. PBS was aspired and the pellet was resuspended in of OLD-T buffer (40mM Tris/HCl, 150mM NaCl, 25mM EDTA, pH 7,5; 500 µl OLD-T buffer/1ml PBS).

4. Proteinase K (Merck) was added to the mix to a final concentration 1 g/l and the mix was incubated for overnight at 55^{o}C.
5. After the incubation, 1 volume of phenol (ROTH) was added and the solution was mixed gently for 3-5 min. The two phases were separated by centrifugation for 5 min at 2500 min^{-1} at room temperature and the aqueous (upper) phase was transfer into the new microcentrifuge tube.
6. 1 volume of phenol-chloroform mix (1:1) was added and the solution was mixed gently for 3-5 min. The two phases were separated by centrifugation for 5 min at 2500 min^{-1} at room temperature and the aqueous (upper) phase was transfer into the new microcentrifuge tube.
7. 1 volume of chloroform (ROTH) was added and the solution was mixed gently for 3-5 min. The two phases were separated by centrifugation for 5 min at 2500 min^{-1} at room temperature the aqueous (upper) phase was transfer into the new microcentrifuge tube.
8. The DNA was precipitated with 2 volumes of 96% ethanol.
9. DNA was transferred into the new 1,5 ml tube with a sterile glass stick, dried for 3-5 min. and dissolved in 50-100 µl of TE buffer (10 mM Tris-HCl (pH 8,0), 1 mM EDTA (pH 8,0), pH 8,0; Maniatis, 1989).

2.2.2. Mutation detection

Mutations were detected by direct sequencing with the MegaBace sequence analyser (GE Healthcare) and DYEnamic ET terminator cycle sequencing kit (GE Healthcare), as it is recommended by its protocol.

The sequencing data were compared with *EFNB1* reference sequence GenBank accession number NM_004429.4 and NG_008887.71 and Ensembl number ENST00000204961. Mutations were confirmed by exon-specific PCR amplification and restriction enzyme digestion in all of the primary cell cultures. PCR primers were 5'-CAAGTTCCTGAGTGGGAAGG-3' and 5'-GTGTGGCCATCTTGACAGTG-3', producing a 455bp fragment containing exons 2-4 for analysis of c.377_384delTCAAGAAG mutation; 5'-GGCTCTTGTCCGCTTCCCTG-3' and 5'-CCAGTCTTCAAAGGGGATCA-3' producing a 502 bp fragment containing exon 2 and intron 2 for analysing c.406+2T>C mutations, primer pair 5'-AGGAACAGTCAGCCAGGGG-3' and 5'-GGGGAGCAGGCGTAGGGTTA-3'

producing a 377 bp product containing exon 4 was used for analysing c.614_615delCT. Primers were designed using the program Primer3 v.0.4.0 http://frodo.wi.mit.edu). Mutations c.377_384delTCAAGAAG and c.614_615delCT were detected by *HinfI* restriction enzyme (New England Biolabs) cleavage of the PCR-products, mutation c.406+2T>C was detected by *BfuAI* restriction enzyme (New England Biolabs) cleavage of the PCR-products. Primers were provided by PD Dr. I. Wieland. Analysis of the restriction fragments was performed using the agarose gel-electrophoresis, 100 bp DNA Ladder by NEW ENGLAND BioLabs was used as a control.

2.2.3. RNA extraction with NucleoSpin® RNA / Protein

Extraction of RNA was performed using the NucleoSpin® RNA / Protein kit (MACHERY-NAGEL) according to the supplier protocol:

1. 10^5 eukaryotic cultured were collected by centrifugation and lysed by directly addition of Buffer RP1.
2. 350 µl Buffer RP1 and 3.5 µl ß-mercaptoethanol (J.T.Baker) were added to the cell pellet and the mix was vortexed vigorously.
3. The mix was filtered through the NucleoSpin® Filter as it is recommended by the supplier: centrifugation for 1 min at 11,000 x g.
4. NucleoSpin® Filter was discarded and 350 µl ethanol (70 %) was added to the flow-through. The mix was pipetted up and down approx. 5 times.
5. For each RNA extraction, one NucleoSpin® RNA/Protein Column was used. It was placed in a collection tube and the lysate was transferred into the column. As it is recommended by the supplier, the column was centrifuge for 30 s at 11,000 x g. After the centrifugation the column was placed in a new collection tube (2 ml, supplied with the kit).
6. 350 µl MDB (Membrane Desalting Buffer) were added into the Column and it was centrifuged at 11,000 x g for 1 min to dry the membrane.
7. Meanwhile the rDNase reaction mixture was prepared in a sterile microcentrifuge tube. For each isolation, as it was recommended by the supplier the DNA restriction mixture was composed as follows: 10 µl reconstituted rDNase and 90 µl Reaction Buffer for rDNase were mixed by flicking the tube gently, 95 µl of the rDNase reaction mixture was applied

directly onto the centre of the silica membrane of the column and the column was incubated at room temperature for 15 min.

8. The NucleoSpin® RNA/Protein Column was washed with 200 µl of Buffer RA2 (centrifugation for 30 s at 11,000 x g). After centrifugation the NucleoSpin® RNA/Protein Column was transferred into a new collection tube (2 ml).
9. The NucleoSpin® RNA/Protein Column was washed with 600 µl of Buffer RA3 (centrifugation for 30 s at 11,000 x g). The flow-through was discarded, the column was placed back into the same collection tube.
10. The NucleoSpin® RNA/Protein Column was washed with 250 µl of Buffer RA3 (centrifugation for 2 min at 11,000 x g to dry the membrane completely). After centrifugation the NucleoSpin® RNA/Protein Column was transferred into an RNase-free Collection Tube (1.5 ml, supplied with the kit).
11. The RNA was eluted with 60 µl RNase-free H_2O (supplied with the kit) by centrifugation at 11,000 x g for 1 min.

2.2.4. RT-PCR and cloning

For expression analysis and generating the p.P54L and p.T111I constructs by *in vitro* site-directed mutagenesis total RNA was reverse transcribed using Super Script™ One-Step™ RT-PCR System (Life Technologies) following the protocol:

1. The PCR reaction was assembled was assembled as follows:

Oligo dT primer (0.5 g/l)	0.5 µl
pd$(N)_6$ Random primer (0.2 g/l)	1 µl
10 mM dNTP Mix	1 µl
RNA	2 µg
MQ H_2O	up to 12 µl

2. The reaction mixture were mixed gently and incubated at 65°C for 5 min.
3. After the 65°C incubation the mix was incubated on ice for 15 min.
4. After the ice incubation following reagents were added into a PCR reaction tube:

5x PCR buffer (200mM Tris-HCl (pH 8.4), 500 mM KCl, Invitrogen)	4 µl

0,1 M DTT (Invitrogen)	2 µl	
RNAse OUT (Invitrogen)	1 µl	
Superscript II (Invitrogen)	1 µl	

5. The reaction was mixed gently and incubated at 42°C for 1 h.
6. After the 42°C incubation, PCR reaction was incubated at 70°C for 15 min.
7. After the 70°C incubation, PCR reaction was put on ice for 15 min for immediate use or frozen.

For analysis of PTC-causing mutations primer pair 5'-CAAGTTCCTGAGTGGGAAGG-3' and 5'-GTGTGGCCATCTTGACAGTG-3' was used to amplify a 455 bp product from exons 2-4, primer pair 5'-GGCTCTTGTCCGCTTCCCTG-3' and 5'-CCAGTCTTCAAAGGGGATCA-3' was used to amplify a 502 bp product from exon 2 and intron 2 and primer pair 5'-ATCATGAAGGTTGGGCAAGA-3' and 5'-TGGGGGCAGTAGTTGTTCTC-3' was used to amplify a 467 bp product from exons 4 and 5 of *EFNB1*. All primers were provided by PD Dr. I. Wieland.

455 bp fragment was constructed using the following program:

1.	94°C	3 min	
2.	94 °C	1 min	40 cycles
	59 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	45 sec	

467 bp fragment was constructed using the following program:

1.	94°C	3 min	
2.	94 °C	1 min	50 cycles
	55 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	45 sec	

502 bp fragment was constructed using the following program:

1.	94°C	3 min	
2.	94 °C	1 min	50 cycles:
	63 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	1 min	

For generating the p.P54L and p.T111I constructs, amplification of *EFNB1* cDNA was performed using 5'-GGCAGAGGAAGGCGAGGCGA-3' and 5'-GCAAGGGGAGGGGGTGTG-3' primer pair that generates a 1,2 Kb product.

1,2 Kb fragment was constructed using the following program:

1.	94°C	2 min	
2.	94 °C	10 sec	10 cycles
	63 °C	30 sec	
	68 °C	2 min	
3.	94 °C	15 sec	10 cycles:
	63 °C	30 sec	
	68 °C	5 min	
4.	72 °C	45 sec	

2.2.5. Real-time RT-PCR

Quantitative RT-PCR analysis was performed using an iQ™ SYBR green supermix kit (Bio-Rad Laboratories GmbH) according to the kit's protocol. Primer pair 5'-CAAGTTCCTGAGTGGGAAGG-3' and 5'-GTGTGGCCATCTTGACAGTG-3' was used to amplify a 455 bp product from exons 2-4 of *EFNB1*, the program is described in section "RT-PCR". Primer pair 5'-TCACAGAGACCCAGGTCAAA-3' and 5'-GGATGGGAAGCACAGGTCTA-3' was used for *MSX2*.

For every real-time PCR reaction the mix was prepared as follows:

1.	SYBR green supermix	12,5 µl
2.	Primer 1 (2 pmol/µl)	1 µl
3.	Primer 2 (2 pmol/µl)	1 µl
4.	DNA	1 µg
5.	MQ water	up to 25 µl

The following program was used for *MSX2* amplification:

1.	95°C	4 min	
2.	95 °C	1 min	50 cycles
	60 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	45 sec	

Primer pair 5'-CTCGGACAAGCTGAGCAAG-3' and 5'-TTTTTAGTTATCCAGCTCCAGAGTC-3' was used for *TWIST1.*

The following program was used for *TWIST1* amplification:

1.	95°C	4 min	
2.	95 °C	45 sec	50 cycles
	58 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	45 sec	

Real-time amplification was performed by iCyler iQ™ Real Time PCR Detection System (Bio-Rad). The threshold cycle number (C_t) was measured using the iCycler and its associated software. To calculate the relative expression of gene of interest, the cDNA from housekeeping gene *G3PDH* was used as a control, primer pair 5'-TGGTATCGTGGAAGGACTCA-3' and 5'-ATGCCAGTGAGCTTCCCGTT-3' (Wieland et al., 1999) was used for the amplification.

The following program was used for *G3PDH* amplification:

1.	94°C	3 min	
2.	94 °C	1 min	50 cycles
	62 °C	45 sec	
	72 °C	45 sec	
3.	72 °C	45 sec	

Gene of interest mRNA expression was normalised with *G3PDH* mRNA expression by calculating $\Delta(\Delta C_t)$ as it is described by Livak and Schmittgen (2001). Relative levels were calculated by the $2^{-\Delta(\Delta Ct)}$ method. Results are given as fold differences to control primary fetal fibroblasts (Livak and Schmittgen, 2001). For each PCR the melting curves were generated to ensure the purity of the amplification product.

2.2.6. Generation of the mutant *EFNB1* cDNA constructs by site-directed *in vitro* mutagenesis

EFNB1 RNA preparation from placenta, reverse transcription and *EFNB1* cDNA amplification were performed as described above. This 1,2 Kb RT-PCR product was cloned into the pCR 2.1 vector (Invitrogen). Mutant *EFNB1* cDNA containing

c.161C>T and c.332C>T mutations were generated using *EFNB1*-specific primers: 5'-GGGCTTGGTGATCTATCTGAAAATTGGAGACAAGC-3' and 5'-GCTTGTCTCCAATTTTCAGATAGATCACCAAGCCC-3' for c.161C>T, 5'-CAGAGCAGGAAATACGCTTTATAATCAAGTTCCAGGAGTTCA-3' and 5'-CTGAACTCCTGGAACTTGATTATAAAGCGTATTTCCTGCTCTG-3' for c.332_333CC>TA (nucleotide exchanges are underlined; primers were designed using the web-based primer software program http://labtools.stratagene.com/QC). Site-directed *in vitro* mutagenesis was performed using QuikChange® II Site-Directed Mutagenesis Kit (Stratagene) according to its protocol:

The following program was used:

1.	95°C	30 sec	
2.	95 °C	30 sec	40 cycles
	55 °C	1 min	
	68 °C	5 min	
3.	68 °C	1 min	

After the PCR reaction a *Dpn I* treatment was performed:

1. PCR mix was incubated at 4°C for 15 min.
2. 1µl of *Dpn I* (10U/µl) was added to each reaction and the mix was incubated at 37 °C for 1 h.

In patient nucleotide exchange c.332C>T leads to the codon exchange ACC>ATC and amino acid exchange threonine to isoleucine. In this work nucleotide exchange c.332_333CC>TA was used. It leads to the codon exchange ACC>ATA and the same amino acid exchange. That was done because the threonine codon ATA is more frequently used then ATC.

After the sequencing, wild type and mutant *EFNB1* cDNA inserts were recloned in pcDNA 3.1(+) vector (BD Biosciences) using *EcoRI* restriction enzyme (MBI Fermentas).

2.2.7. Sequencing

The presence of mutations was confirmed by sequencing using ALFExpress sequencing machine (Amersham Biosciences) and the Cy5™ AutoRead™ Sequencing Kit (Amersham Biosciences) according to the modified kit's protocol:

I. The standard annealing of primer to double-stranded template was performed.

1. Mix with the following reagents was assembled in the 1,5 microcentrifuge tube:

Template DNA	32 µl
2 M NaOH	8 µl
Total volume	40 µl

2. The mix was vortexed gently, than briefly centrifuged and then incubate at room temperature for 10 min.
3. After the incubation 7 µl of 3 M sodium acetate (pH 4.8), 4 µl of dH_2O and 120 µl of 100% ethanol were added into the tube. The reaction was mixed and frozen for 15 min.
4. After that the DNA was precipitated by centrifugation for 30 min at 10 000 min^{-1} at 4^oC.
5. The supernatant was discarded and the pellet was rinsed with 70% ethanol. The pellet was recentrifuged again for 15 min, 10 000 min^{-1}, 4^oC, and the supernatant was discarded. The pellet was dried for 5 min under the medium vacuum.
6. Dry pellet was resuspended in 10 µl of distilled water.
7. Then the following reagents were added to the resuspended pellet:

Cy5 Primer (4-10 pmol)	2 µl
Annealing buffer	2 µl
Total volume (including the resuspended pallet)	14 µl

8. The mix was gently vortexed and briefly centrifuged. The annealing reaction was incubated at 65^oC for 5 min. Immediately after the 65^oC incubation, the reaction was placed at 37^oC and incubated for 10 min. After that the tube was placed at room temperature for some time (longer than 10 min).

   During the room temperature incubation wells of the MicroSample Plate (NUNC) was labelled “A”, “C”, “G” and “T” respectively, 2,5 µl of the “A” Mix, “C” Mix, “G” Mix and “T” Mix were pipetted into the corresponding labelled wells, respectively, (all procedures were done at 4^oC).
9. Annealing reaction was centrifuged briefly and 1 µl of Extension Buffer and 3 µl of DMSO were added into the tube. Immediately after that the sequencing reaction was started.

II. Sequencing Reaction.

1. MicroSample Plate with “A”, “C”, “G” and “T” Mixes were placed in a water bathe (37^{o}C) for at least 1 min. All the following steps were performed at 37^{o}C.
2. 2 µl of diluted T7 DNA Polymerase were added to the annealing reaction and the reagents were mixed thoroughly with a pipette tip. Immediately after that 4.5 µl of this mixture were transferred into each of the prewarmed sequencing mixes and incubated for 5 min.
3. 5 µl of Stop Solution were added to each reaction and the reaction was mixed by gentle agitation. After adding the Stop Solution the reaction was heated to 88^{o}C for 3 min and then immediately quenched on ice; 5 µl of “A”, “C”, “G” and “T” sequencing reactions were loaded into the appropriate wells of a sequencing gel.

2.2.8. Western blot analysis

Patient fibroblasts were lysed with RIPA buffer (30 mM Tris-HCl (pH 7,5), 0,12 M NaCl, 10% Glycerol and 1% Triton X100 as described by Harlow and Lane, 1988) and used for the SDS-PAGE Western blot analysis. Gel-electrophoresis was performed using MINI PROTEAN II™ electrophoresis devise (BIO-RAD) and 10% polyacrylamide TRIS-HCl gels (BIO-RAD). Blotting was performed in TRANS-BLOT® SD SEMI-DRY TRANSFER CELL (BIO-RAD) using the PVDF Transfer Membrane (HEH™ Life Science Product Inc.). Anti-ephrin-B1 antibody (A-20, Santa Cruz Biotechnology, Inc.) was used at a ratio 1:200 as it is recommended by the supplier, incubation was overnight at 4^{o}C. As a control a Precision Plus Protein Standard Dual Color (BIO-RAD) was used.

NIH 3T3 cells were washed with PBS and lysed in RIPA buffer containing PhosphoStop solution (1 tablet per 10 ml of RIPA buffer, Roche) immediately after the EphB2-Fc stimulation. Lysates were kept on ice and used for the SDS-PAGE Western blot analysis. Phospho-Ephrin B (Tyr324/329) antibody (Cell Signaling Technology®) was used at a ratio 1:1000 as it is recommended by the supplier, incubation was overnight at 4^{o}C, and anti-ephrin-B1 antibody (A-20) was used to detect ephrin-B1. Visualizer™ Western Blot Detection Kit (UPSTATE) was used according to the protocol for visualization of the bands:

1. Detection reagents A and B were mixed at a ratio 1:2 respectively (final volume was approximately 3 ml for a single mini-gel sized blot, 3 ml per 50 cm^2 of membrane) and incubated for 1 h at room temperature.
2. After the incubation, mix was pipetted onto the blotted membrane and incubated for 5 minutes at room temperature
3. The membrane was removed from the mix, rinsed with MQ water and placed into the Saran wrap.
4. The membrane was exposured with the X-ray film, exposure time is 5”-3’.

All experiments were performed at least twice to reproduce the results.

3. Results

3.1. Molecular analysis of the PTC-causing EFNB1 mutations in patient fibroblasts

3.1.1. Frameshift mutation c.377_384delTCAAGAAG

In this work three PTC-causing mutations were analysed. All three were predicted to be protein-truncating mutations. But usually PTC lead to the NMD, that is why first the mRNA level of these mutations was investigated.

Frameshift mutation c.377_384delTCAAGAAG generates a premature termination codon (PTC) 17 nucleotides upstream of exon 2/exon 3 junction. RT-PCR analysis showed that primary patient fibroblast culture has very low level of the mutant *EFNB1* expression. One possible explanation was preferentially inactivation of the mutant

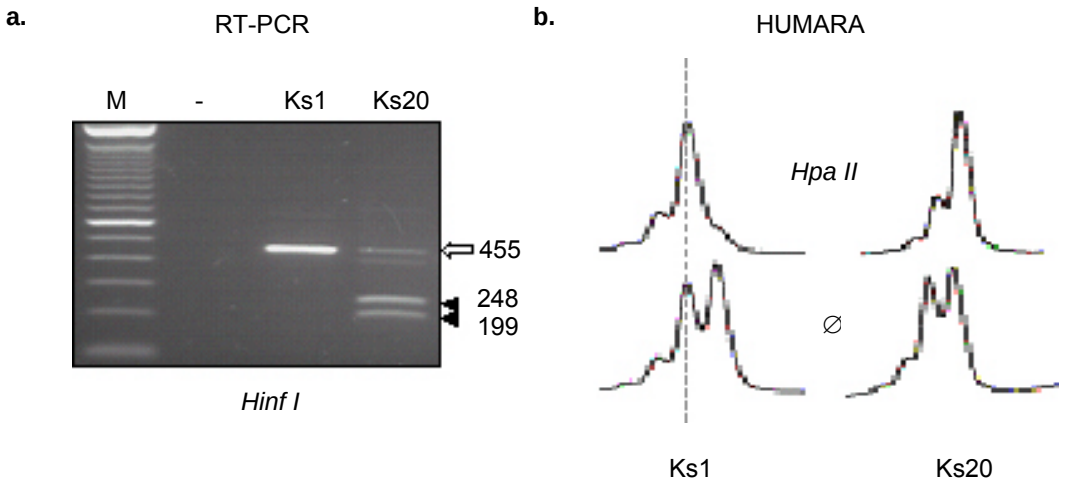


Fig. 3. Analysis of c.377_384delTCAAGAAG fibroblasts. **a.** RT-PCR analysis of *EFNB1* transcripts expressed in subclones of primary patient fibroblasts. RT-PCR products were digested by *Hinf I*. Cell clone Ks1 showed expression of the wild type allele, (455 bp fragment, indicated by arrow), whereas Ks20 showed only a very low level of mutant allele (digested into the 199 bp and 248 bp fragments, indicated by arrowheads). Band with the size ≈455 bp that was detected in Ks20 appeared to be the unspecific one. **b.** HUMARA analysis of Ks1 and Ks20 clones. A clonality of Ks1 and Ks 20 was demonstrated. Ks1 has one X-chromosome inactiveted whereas Ks20 has another X-chromosome inactived.

EFNB1 carrying X-chromosome, another explanation was degradation of the mutant *EFNB1* transcript. HUMARA showed random X-inactivation in the primary culture (not

shown) that excluded the preferentially inactivation of the mutant *EFNB1* carrying X-chromosome. To analyse the possibility of mRNA degradation, fibroblasts were subcloned into several subclones. Clonality of two of them, Ks1 and Ks20 was proven by HUMARA (**Fig. 3b**). It showed that in Ks1 one X-chromosome is inactivated whereas in Ks20 another X-chromosome is inactivated (**Fig. 3b**). The results of HUMARA are provided by PD Dr. I. Wieland. RT-PCR with following *Hinf I* digestion showed that one of these clones – Ks20 expresses only the mutant allele (447 bp RT-PCR product, digested by *Hinf I* into 199 bp and 248 bp fragments) while Ks1 clone express only the wild type *EFNB1* allele (455 bp RT-PCR product). Also Ks20 appeared to have a very much lower level of *EFNB1* expression (**Fig. 3a**). To measure the decrease of *EFNB1* expression level, a real-time PCR was performed (**Fig. 4**). Fibroblasts were harvested for the RNA extraction using the NucleoSpin® RNA / Protein kit (MACHERY-NAGEL). To exclude the mistakes caused by the natural variations of the genes expression a housekeeping gene *G3PDH* was used as a control. Real-time PCR showed that primary patient fibroblasts have ≈8,42%, Ks1 clone has ≈8,85% and Ks20 has only ≈0,55% of *EFNB1* expression compared to normal fibroblasts. It was proposed that decrease of *EFNB1* expression level is caused by nonsense-mediated mRNA decay (NMD, Wieland et al., 2008).

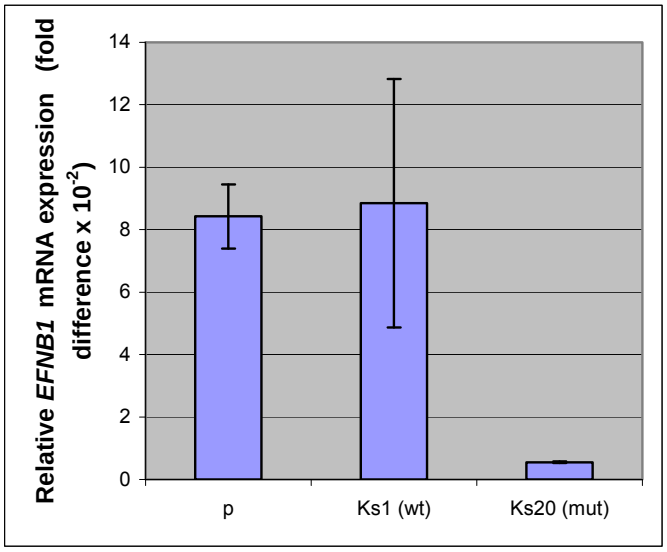


Fig. 4 Real-time PCR analysis of c.377_384delTCAAGAAG subclones Ks1 and Ks20. Fibroblasts were used for the RNA extraction, RNA was reverse transcribed to cDNA and used for the real-time PCR. Primary patient fibroblasts were shown to have strong decrease of the *EFNB1* expression compared to normal fibroblasts.

3.1.2. Frameshift mutation c.614_615delCT

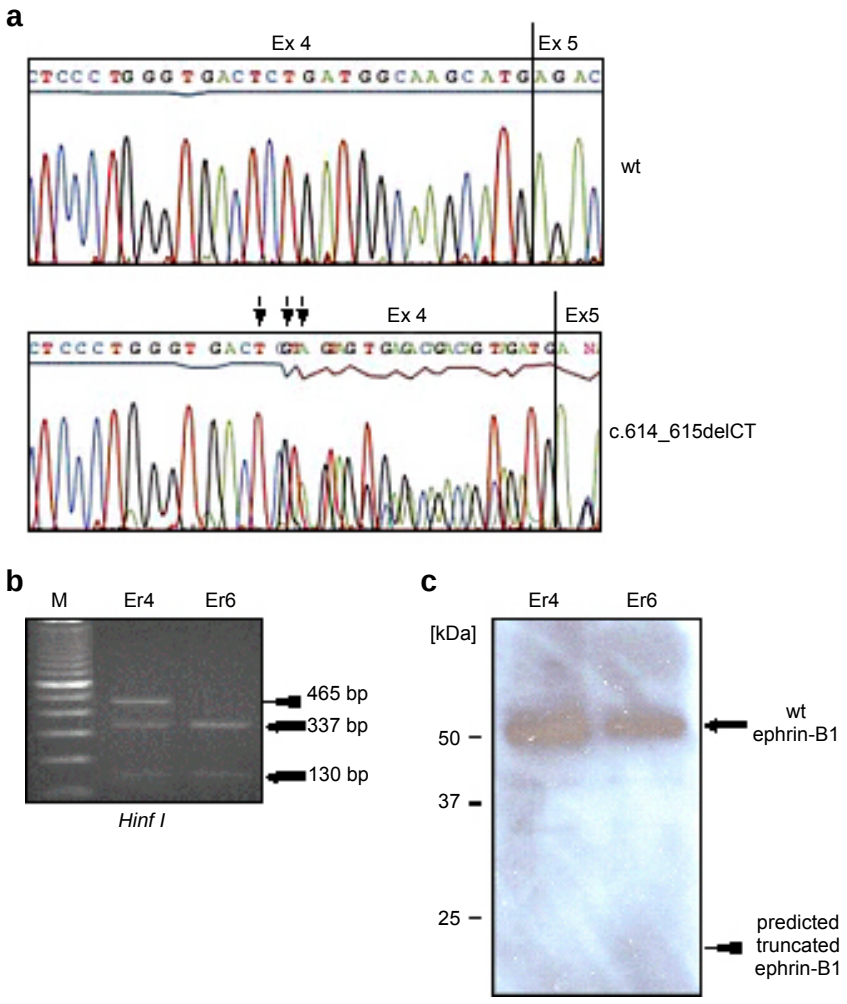


Fig. 5 Expression of *EFNB1* transcript and protein in primary patient fibroblasts harbouring heterozygous frameshift mutation c.614_615delCT. **a** Sequence analysis of the patient fibroblats. Uprer panel shows the wild type allele, lower panel shows the mutant allele. Deletion of the CT dinucleotide creates a premature termination codon TGA in exon 4 (indicated by arrows). **b** RT-PCR analysis of Er4 and Er6 clones. Er4 clone appeared to be expressing both wild type and mutant *EFNB1* alleles whereas clone Er6 express only wild type *EFNB1* allele. Wild type and mutant RT-PCR products were digested by the restriction enzyme *Hinf I*. Wild type is digested to the 130 bp and 337 pb fragments (indicated by arrows), mutant allele is undigested and indicated by an arrowhead (465 bp). Size markers are shown in lane M (100 bp DNA ladder, Invitrogen). **c** Western blot analysis of ephrin-B1 expression in patient fibroblasts culture. Wild type ephrin-B1 (≈50 kDa) was detected in both Er4 and Er6 (indicated by an arrow). No smaller truncated protein corresponding to c.614_615delCT was detected at the predicted molecular weight of ≈20 kDa in Er4 (expected size indicated by an arrowhead) using an anti-ephrin-B1 antibody.

Frameshift mutation c.377_384delTcAAGAAG was shown to cause transcript depletion by NMD (Wieland et al., 2008). However, escape from NMD was shown for c.614_615delCT mutation. This mutation is located in exon 4 of *EFNB1* and cause PTC in 14 nucleotides upstream of 3'-end of the exon 4 (Wieland et al. 2008).

In patient fibroblast cultures, RT-PCR revealed a mutant *EFNB1* transcript in addition to the wild type transcript (**Fig. 5a**). In contrast to the c.377_384delTcAAGAAG mutation, the level of the c.614_615delCT mutant allele expression is not highly reduced. For the further investigation patient fibroblasts were recloned into several subclones. The results of the HUMARA were not informative (not shown). To distinguish wild type and mutant transcripts, RT-PCR analysis of the subclones was performed, the RT-PCR products were digested by *Hinf I* restriction enzyme. It was found out, that some of the analysed c.614_615delCT subclones (e.g. Er6) express only the wild type *EFNB1* allele (467 bp RT-PCR product, digested into two fragments: 130 nucleotides and 337 nucleotides), whereas other subclones (e.g. Er4) are mixed i.e. they expresses both wild type and mutant (465 bp RT-PCR product) alleles (**Fig. 5b**).

As it was told before, PTC containing mRNA was predicted to give rise to the truncated, soluble ephrin-B1 protein. To detect truncated ephrin-B1, proteins from Er4 and Er6 subclones were extracted and Western blot analysis was performed (**Fig. 5c**). In both subclones wild type ephrin-B1 (≈50 kDa) was detected as it was expected according to the results of the RT-PCR analysis. But the expected truncated protein (≈20 kDa) was not found in Er6 subclone that express both wild type and mutant *EFNB1* alleles.

3.1.3. Splice-site mutation c.406+2T>C

In another girl from unaffected parents, *de novo* occurrence of the heterozygous splice-site mutation c.406+2T>C was detected in the genomic DNA (**Fig. 6a**, right panel). Splice-site mutation c.406+2T>C alters the consensus splice donor site "GT" of exon 2 at the junction of exons 2 and 3.

Analysis of the patient fibroblasts by RT-PCR revealed that the wild type *EFNB1* transcript was the main transcript, but additional transcripts were derived from the splice-site mutation allele c.406+2T>C (**Fig. 6b**). This mutation leads to retention of intron 2 (corresponds to the 288 bp fragment, the **Fig. 6b** right panel) or to the

activation of a cryptic splice site within exon 2 (corresponds to the ≈1,2 Kb fragment, **Fig. 6b** right panel). Presence of the both splice variants has been confirmed by

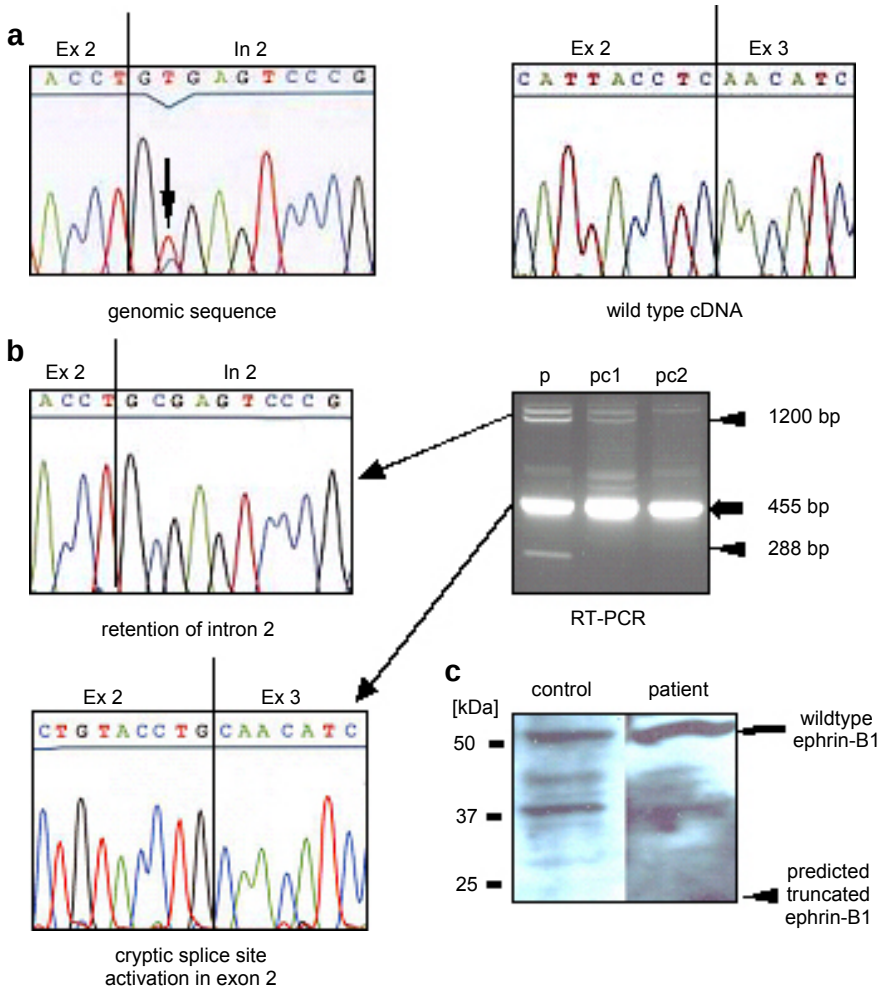


Fig. 6 Expression of *EFNB1* transcript and protein in primary patient fibroblasts harbouring splice site mutation c.406+2T>C. **a** Sequence analysis of the patient genomic DNA. Nucleotide exchange T>C in intron 2 at the splice donor site is indicated by an arrow (left panel). The major transcript expressed in patient fibroblasts was the wild type allele (right pannel). **b** RT-PCR analysis of the transcripts. Wild type and mutant transcripts in patient fibroblasts (lane p) and control cell cultures (lanes pc1 and pc2) were determined by RT-PCR. The wild type RT-PCR product is indicated by an arrow, mutant RT-PCR products are indicated by arrowheads. Sequencing of the aberrant transcripts showed retention of intron 2 or activation of a cryptic splice site in exon 2 (left pannels). **c** Western blot analysis of ephrin-B1 expression in patient fibroblasts and control cell culture lysates showed an approximately 50 kDa protein (indicated by an arrow). No smaller truncated protein was detected at the expected molecular weight of ≈10 kDa in patient fibroblasts. Protein sizes were determined using Precision Plus Protein™ Standards Dual Color (BIO-RAD).

cDNA cloning and sequencing analysis (**Fig. 6b** left panel).

Both splicing variants resulted in PTCs and were predicted to cause a truncated ephrin-B1 protein. To detect the truncated proteins, Western blot analysis was performed (**Fig. 6c**). As in case of c.614_615delCT mutation, the wild type ephrin-B1 (≈50 kDa) but no truncated protein (≈10 kDa) was detected. Also Western blot revealed the band with ≈38 kDa size in both patient and control (transfected NIH 3T3) cells. This band has been detected several times. It was proposed that this band corresponds to the ephrin-B1 protein digested with γ-secretase (see above) or to the unglycosylated ephrin-B1.

3.2. Analysis of missense mutations in a cell culture model

3.2.1. Generation of c.161C>T/p.P54L and c.332_333CC>TA/p.T111I expression constructs and establishing the NIH 3T3 cell culture model

The second most frequent type of mutation found in CFNS patients is missense mutations in exons 2 and 3 of *EFNB1*. Missense mutation c.161C>T in exon 2 has been recurrently detected in familial and sporadic CFNS patients. This mutation results in proline to leucine amino acid exchange in position 54 (p.P54L) of the extracellular ephrin domain (Wieland et al. 2004). Missense mutation c.332C>T is also located in exon 2 and has been previously described in a large German CFNS family (Wieland et al. 2004). This mutation changes threonine for isoleucine at ephrin-B1 position 111 (p.T111I) which maps to the G-H loop at the high-affinity Eph/ephrin interface (Nikolov et al. 2005). To determine the role of these missense mutations in EphB2/ephrin-B1 signalling and cell behaviour, a cell culture model was established. Expression constructs containing wild type, c.161C>T/(p.P54L) and c.332CC>TA/(p.T111I) *EFNB1* cDNA, respectively, were generated by site-directed mutagenesis and used for transfection of NIH 3T3 cells (**Fig. 7a**). NIH 3T3 were chosen while they do not express mouse homologues of B-type ephrin genes (Moreno-Flores et al. 2002 and our results, **Fig. 7b**, first well). Transfection efficiency of the constructs in NIH3T3 was monitored by RT-PCR and FACS analysis (**Fig. 7b**, **c**).

3.2.2. The role of p.P54L and p.T111I mutations in cell behaviour: patches formation analysis

It is known, that the EphB2/ephrin-B1 system is important for the cell behaviour, adhesion and repulsion (Klein et al. 2004). The role of EphB2 expressing cells in these processes has been intensively investigated (Stein et al. 1998; Zimmer et al. 2003). The role of ephrin-B1 expressing cells, however, appears to be less clear. To understand the impact of missense mutations on cell behaviour, wild type, p.P54L and p.T111I ephrin-B1 expressing NIH3T3 cells were stimulated with preclustered

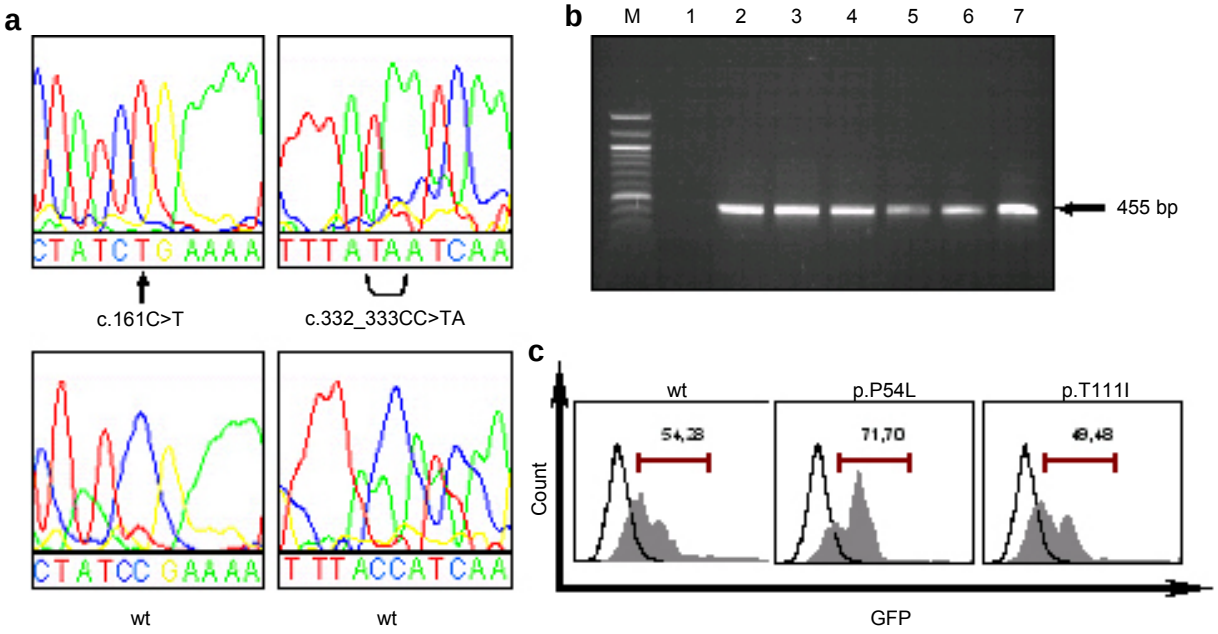


Fig. 7 Generation of c.161C>T and c.332CC>TA *EFNB1* cDNA constructs by site-directed mutagenesis and transfection into NIH 3T3. **a** Sequence analysis of the *EFNB1* cDNA constructs (exon 2) after the site-directed mutagenesis. The *upper panel* for each mutation represents the result of the mutagenesis while the *lower panel* represents the wild type *EFNB1* cDNA. Nucleotide exchanges are indicated by arrow and bracket. **b** RT-PCR using *EFNB1* specific primers was performed. RT-PCR products of the primary NIH 3T3 cells (lane 1), NIH 3T3 cells tranfected with wild type, c.161C>T (p.P54L), c.332 CC>TA (p.T111I) *EFNB1* cDNA constructs, respectively (lanes 2-6) and Cos-1 cells (lane 7). Size markers are shown in lane M (100 bp DNA ladder, Invitrogen) **c** FACS analysis of the NIH 3T3 cells transfection efficiency. Grey peaks show maximum of GFP fluorescence in NIH 3T3 transfected cells. Empty peaks show maximum of GFP fluorescence in untransfected control cells.

EphB2-Fc for 30 min. After the stimulation cells were cultivated for 24 h and than pictures of the cells were taken using the fluorescence microscopy method. Pictures showed that wild type and p.T111I ephrin-B1 expressing NIH3T3 cells formed clusters (**Fig. 8a**), whereas cells expressing p.P54L ephrin-B1 were scattered much like the ephrin-B1 expressing cells in the control following Fc-only treatment (**Fig. 8a**) or like the ephrin-B1 – expressing cells before the stimulation (not shown).

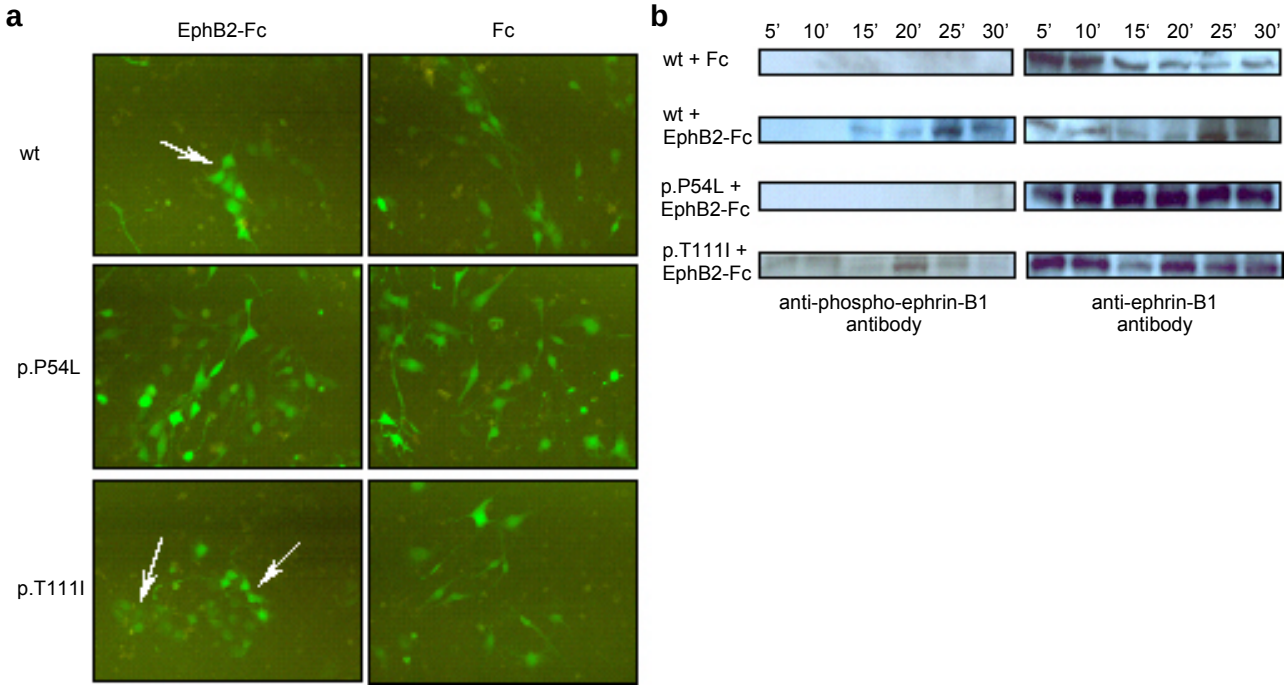


Fig. 8 EphB2-Fc stimulation of wild type, p.P54L, p.T111I ephrin-B1 expressing NIH 3T3 cells. **a** Right panel: NIH 3T3 cells expressing wild type and p.T111I eprin-B1 were detected by fluorescent microscopy as cell clusters after the EphB2-Fc stimulation (indicated by arrows). No patches was detected in the p.P54L expressing cells. Left panel: the same cell lines treated with Fcwere, cells do not form any patches. **b** Western blot analysis of the wild type, p.P54L and p.T111I ephrin-B1 expressing NIH 3T3 cells. Cells were stimulated for 5 to 30 min with EphB2-Fc, proteins were extracted. Western blot was performed using the Phospho-Ephrin B (Tyr324/329) antibody and anti-ephrin-B1 antibody. NIH 3T3 cells transfected with wild type ephrin-B1 and treated with Fc were used as a control. Protein sizes were determined using Precision Plus Protein^{TM} Standards Dual Color (BIO-RAD).

3.2.3. The role of p.P54L and p.T111I mutations in ephrin-B1 phosphorylation

Reverse signalling of ephrin-B1 involves phosphorylation of several conserved tyrosine residues of the cytoplasmic tail, including Tyr324 and Tyr329 (Kalo et al. 2001). To determine the impact of c.161C>T and c.332C>T missense mutations on this Tyr-phosphorylation, the timing Tyr324 and Tyr329 of wild type and mutant ephrin-B1 was investigated. NIH 3T3 cells expressing c.161C>T and c.332C>T *EFNB1* were stimulated with preclustered EphB2-Fc from 5 to 30 min. After the stimulation, Tyr324 and Tyr329 phosphorylation response was monitored by Western blot analysis using Tyr324/329-specific polyclonal antibodies (**Fig. 8b**). Phosphorylation status was checked after 5, 10, 15, 20, 25 and 30 minutes of stimulation. This showed phosphorylation of the wild type ephrin-B1 began to be detectable approximately at 15th min after the stimulation and prolonged to at least 30th minute. In p.T111I mutant ephrin-B1 expressing NIH3T3 cells, phosphorylation appeared to be detectable approximately at 20th minute of stimulation and prolonged for a shorter period of time, approximately until 25th minute of stimulation. For p.P54L mutant ephrin-B1 no phosphorylation was detected like in the control wild type ephrin-B1 expressing cells treated with Fc only.

3.3. Expression analysis of Msx2 and Twist1 genes

After the analysis of wild type and mutant ephrin-B1 phosphorylation, it was decided to have a look at the other members of ephrin-B1 reverse signalling.

It was proposed that ephrin-B1 stimulation with EphB2-Fc would change the expression level of *Msx2* and *Twist1* genes via ephrin-B1 – Stat3 interaction. To measure the expression level of genes, a real-time PCR analysis was performed. Cells expressing wild type, p.P54L and p.T111I ephrin-B1 were stimulated with EphB2-Fc for 30 min. After the stimulation NIH 3T3 fibroblasts were harvested for the RNA extraction. This RNA was reverse transcribed into the cDNA that was used for the real-time PCR. To calculate the relative *Msx2* and *Twist1* expression, a housekeeping gene *G3PDH* was used as a control to exclude the mistakes caused by natural expression variation between the cell lines. In this experiment only the expression change exceeding the 2 fold was used as a relevant change. This was

done to exclude the possible mistakes caused by minor variations of transfection rate and pipetting.

It was already known that p.P54L rather had loss-of-function effect. No change either of *Twist1* or *Msx2* expression level was expected and only the minor, not exceeding the 2 fold was detected. Also, only the minor *Twist1*/*Msx2* expression level change was detected in p.T111I expressing sells. Surprisingly, also no exceeding the 2 fold changes in expression level of genes of interest was detected in cells transfected with wild-type ephrin-B1 (**Fig. 9**).

Cut-off of the fold difference less than 2 was chosen according to the $2^{-\Delta(\Delta Ct)}$ formula. Such cut-off means that the minimal difference between the threshold cycles of the genes of interest must be at least 1 cycle. The cut-off helps to avoid the pipetting and instrumental mistakes.

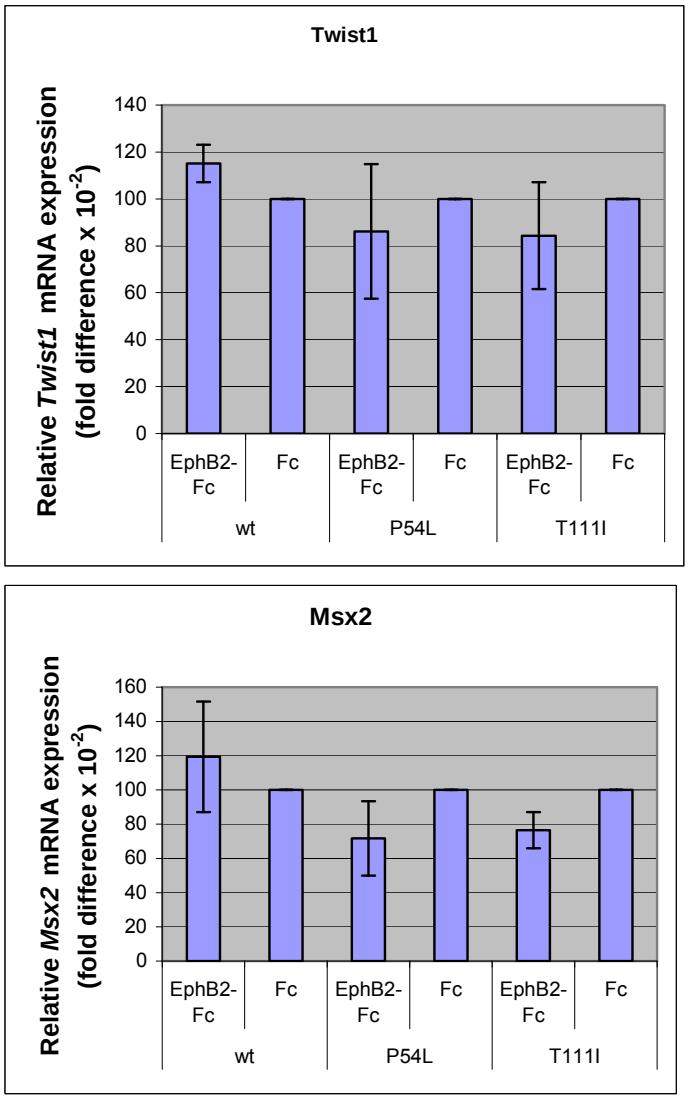


Fig. 9 Real-time PCR analysis of *Twist1* and *Msx2* in ephrin-B1 expressing NIH 3T3 cells. Cells were transfected and stimulated with EphB2-Fc for 30 min as it is described before. Stimulated cells were used for the RNA extraction, RNA was reversed transcribed to cDNA and used for the real-time PCR. As it was expected, no change of *Twist1* and *Msx2* expression level was detected in p.P54L. No change of *Twist1* and *Msx2* expression level was detected in p.T111I. Surprisingly, no change of *Twist1* and *Msx2* expression level was detected in wild type ephrin-B1 expressing cells.

4. Discussion

4.1. PTC-causing mutations

4.1.1. Frameshift mutation c.377_384delTCAAGAAG

In this study, we examined the impact of disease-causing mutations in the *EFNB1* gene.

Frameshift mutation c.377_384delTCAAGAAG was described in a patient with classical CFNS. This mutation is located in exon 2 and leads to numerous PTCs, first of them appears 17 nucleotides upstream the 3'-end of exon 2 (Wieland et al., 2008). Subcloning of the patient fibroblasts primary culture and HUMARA of the subclones showed that two populations of cells with the different active alleles of *EFNB1* exist and can be separated *in vitro*. Carrel and Willard (2005) have shown that approximately 15% of the X-chromosome located genes escape the X-linked inactivation. Inactivation of *EFNB1* was shown to depend from the X-chromosome inactivation pattern. Expression analysis of the patient fibroblasts showed the strong decrease of the mutant *EFNB1* allele expression level, probably, due to NMD. According to that, c.377_384delTCAAGAAG mutation rather had loss-of-function effect.

Previously, it has been shown *in silico* and *in vitro* that CFNS causing mutations disrupt or alter the ephrin-B1 function (Nikolov et al. 2005). The major types of mutations include frameshift, nonsense and splice site mutations that generate premature termination codons. PTC containing mRNA could give rise to truncated, soluble ephrin-B1 polypeptides that lack the transmembrane and intracellular domains. These soluble peptides could be secreted from the mutant *EFNB1* expressing cells and could interact with EphB-receptors expressing cells located far from the *EFNB1* expressing cells altering and disrupting the EphB-receptor forward signalling. Truncated ephrin-B1 polypeptides could exhibit dominant-negative or gain-of function effects.

However, usually PTCs lead to nonsense-mediated mRNA decay. NMD generally involves nonsense codon recognition by translating ribosomes at a position ≈25 nucleotides upstream of a splicing-generated exon junction complex of proteins. As such, NMD provides a means to degrade abnormal mRNAs that encode potentially

deleterious truncated proteins. Additionally, an estimated one third of naturally occurring, alternatively spliced mRNAs is also targeted for NMD (Hillman et al., 2004; Lewis et al., 2003; Mendell et al., 2004). Given the extraordinary frequency of alternative splicing together with data indicating that naturally occurring transcripts other than alternatively spliced mRNAs are likewise targeted for NMD, it is expected that mammalian cells routinely utilize NMD to achieve proper levels of gene expression (Lejeune and Maquat, 2005). In mammalian cells NMD generally occurs when a nonsense codon resides >50–55 nucleotides upstream of a splicing-generated exon-exon junction and is followed by at least one intron (Nagy and Maquat, 1998). Also, it was shown that some genes such as natural intronless genes mouse histone H2a and human histone H4 are resistant to NMD (Maquat and Li, 2001). NMD also was described to play a role in cancer development (Culbertson, 1999). Chain-termination mutations that reduce mRNA abundance by reducing the half-life of mRNA typically behave like loss-of-function alleles. For example, a recessive form of β-thalassemia (OMIM 141900) common in Mediterranean populations results from a mutation that generates a premature UAG stop codon in the *HBB* gene (OMIM 141900). This leads to severely reduced haemoglobin-β mRNA accumulation (Baserga and Benz, 1992).

4.1.2. Frameshift mutation c.614_615delCT and splice-site mutation c.406+2T>C

Frameshift mutation c.614_615delCT has been previously described in a sporadic female patient with CFNS including coronal craniosynostosis, duplex kidneys and diaphragmatic hernia (Wieland et al. 2008). This di-nucleotide deletion occurred in exon 4 of *EFNB1* and generates a PTC in 14 nucleotide upstream of 3'-end of the exon 4. This mutation escapes the NMD according to the "55" bp rule. Expression analysis of patient fibroblasts revealed transcripts from mutant and wild type alleles at similar amounts, whereas only the wild type but no truncated ephrin-B1 was detected by Western blot analysis. This rather suggests that truncated ephrin-B1 proteins are instable and do not contribute substantially to the CFNS phenotype. In this case mutant and wild type *EFNB1* transcripts presented at similar amounts but nevertheless amount of the mutant protein was drastically decreased and the c.614_615delCT seemed to have a loss-of-function effect.

In another female patient with classical CFNS phenotype, splice site mutation c.406+2T>C was detected. Mutations at this splice donor site have been recently described in two independent families (Wallis et al. 2008). In the patient examined here c.406+2T>C occurred *de novo*. In general, splice site mutations constitute about 10% of CFNS point mutations, which is within the range observed in other human genetic diseases (Krawczak et al. 1992). Expression analysis of the patient's fibroblasts revealed retention of intron 2 as has been previously detected for the splice acceptor "AG" mutation c.407-2A>T at the same exon junction (Wieland et al. 2008). In addition, activation of a cryptic splice site in the preceding exon 2 was detected for c.406+2T>C, apparently a frequent consequence of 5' splice site mutations (Buratti et al. 2007; Krawczak et al. 1992). Both, intron retention and cryptic splice site activation resulted in PTCs and reduced transcript amounts when compared with the wild type allele. Like in case of c.614_615delCT mutation, no truncated soluble ephrin-B1 polypeptide was detected in native patient fibroblasts.

As it was already mentioned, mutation c.377_384delTCAAGAAG leads to the strong reduction of the mutant *EFNB1* transcription level probably due to NMD. However, some chain-termination mutations confer a dominant phenotype when the RNA-surveillance system is bypassed. The most clearly defined bypass mechanism involves the skipping of constitutively spliced exons. For example, exon skipping associated with a nonsense mutation has been documented the *FBN1* (fibrillin) gene (OMIM 134797) causing Marfan syndrome (OMIM 154700, Dietz and Kendzior, 1994). Nonsense-associated exon skipping probably occurs when a nonsense mutation is fortuitously located at a position that affects splice-site selection through changes in RNA structure.

Frameshift mutation c.614_615delCT and splice site mutation c.406+2T>C, both, were predicted to result in protein truncation preceding the transmembrane domain of ephrin-B1. This stimulated us to investigate whether the observed mutant transcripts will give rise to a truncated soluble ephrin-B1 protein product. Such protein may exhibit dominant-negative or gain-of-functions effects. To determine, whether mutant transcripts give rise to a truncated ephrin-B1 protein, Western blot analysis was performed. In contrast to presence of wild type and mutant RNA transcripts in patient fibroblasts, only wild type but not a mutant truncated ephrin-B1 protein was detected in both c.614_615delCT and c.406+2C>T fibroblast cultures. It was proposed, that truncated ephrin-B1 is rapidly degraded in the patient fibroblasts. We proposed that

the protein was synthesized and degraded rather than not synthesized because it is known, that rapid degradation of newly synthesized proteins is a routine process. It was shown, that up to 30 % of all newly synthesized proteins are defective and were degraded immediately after the synthesis (Schubert et al., 2000).

Absence of the mutant protein shows that mutations c.614_615delCT and c.406+2C>T appear to have a loss-of-function effect. Frameshift mutation c.377_384delTCAAGAAG also seems to have loss-of-function effect. According to our results and previously published data, we conclude that the major part of all PTC-causing mutations has neither dominant-negative, nor gain-of-function effects but rather loss of function effect.

Plenty of dominant-negative mutations of different genes are known up to now. One of these genes is *SOX10* (OMIM 602229). This gene encodes the transcription factor SOX10. It was shown by Kuhlbrodt et al. (1998) and Pingault et al. (1998) that mutations of this gene cause Waardenburg-Shah syndrome (WS4, OMIM 277580). Several dominant-negative mutations of this gene have been described including the protein-truncating mutations (Sham et al., 2001). This mutation cause more severe disease manifestation than loss-of-function mutations.

Why in case of *SOX10* the truncated protein exists and causes a dominant-negative effect while ephrin-B1 truncated protein is rapidly degraded? It is known that C-terminal part of ephrin-B1 plays an important role in the glycosylation and transporting of the protein to the Golgi apparatus and further to the membranes (Compagni et al. 2003; Davy et al. 2004, 2006). In contrast, C-terminal part of SOX10 does not have any point that can be crucial for the protein transporting.

4.2. Missense mutations p.P54L and p.T111I

Mutation c.161C>T/p.P54L was found in a family with the mother and a daughter affected by craniofrontonasal syndrome and the maternal hemizygous grandfather (Wieland et al., 2004). The grandfather showed only slight facial asymmetry and a broad nasal bridge. His daughter showed severe facial asymmetry, hypertelorism, and hypoplasia of the corpus callosum. The granddaughter exhibited facial asymmetry, hypertelorism, agenesis of the corpus callosum, complete syndactyly of the third and fourth finger on the left side, and scoliosis. This mutation was also described by Twigg et al. (2006) in a familiar case with a mosaicism in the mother of

affected son and daughter. The female proband had classical CFNS and her brother had hypertelorism and a nasal pit, but their mother did not show any CFNS features apart from slight nasal asymmetry and longitudinal grooves in her nails. Wieland et al. (2005) described this mutation in a sporadic case.

Mutation c.332C>T/p.T111I was found in six affected females and three male carriers of a family over five generations (Wieland et al., 2004). The phenotypes of the female patients included hypertelorism, orbital asymmetry, brachycephaly, brachydactyly, and Sprengel deformity. One affected female experienced four miscarriages in midpregnancy and was found to have an uterus arcuatus. In addition she had curly hair, grooved fingernails, and unilateral breast hypoplasia.

Missense mutation p.P54L presumably leads to a conformational change of the extracellular ephrin domain and p.T111I specifically alters the G-H loop involved in Eph receptor - ephrin-B1 recognition (Nikolov et al. 2005). Since c.161C>T mutants is located at the border between B and H β-strands of the extracellular domain, it is unlikely to result in unstable protein (Mellitzer and Wilkinson, 1999; Wieland et al., 2008). Rather, p.P54L mutation disturbs the ligand receptor clustering in cell–cell contacts.

Missense mutation c.332C>T is also located in exon 2. It changes threonine for isoleucine at ephrin-B1 position 111 (p.T111I) which maps to the G-H loop at the high-affinity Eph/ephrin interface (Nikolov et al. 2005). Both missense mutations replace amino acid residues are highly conserved in the three human ephrin-B ligands and across a great variety of species, they were predicted to compromise the biological activity of ephrin-B1 (Nikolov et al. 2005; Wieland et al. 2004).

It has been shown previously, that upon Eph receptor – ephrin-B1 interaction, reverse signalling proceeds through phosphorylation of six tyrosine residues (corresponding to human Tyr313, Tyr317, Tyr324, Tyr329, Tyr343 and Tyr344) in the intracellular part of ephrin-B1 (Chong et al. 2000). Addition of recombinant extracellular domain of EphB2 to ephrin-expressing cells in tissue culture similarly caused tyrosine phosphorylation of the ephrin (Brückner et al. 1997; Holland et al. 1996). Phosphorylation of Tyr 324 and 329 by Src kinases was shown to be the most important for ephrin-B1 reverse signalling (Kalo et al. 2001; Lee et al. 2008; Tanaka et al. 2005). Western blot analysis was performed to detect phosphorylation of Tyr324 and Tyr329 of wild type, p.P45L and p.T111I proteins in response to EphB2-Fc receptor stimulation. For p.P54L mutant ephrin-B1 no phosphorylation was

detected. In contrast, p.T111I ephrin-B1 still showed Tyr324/329 phosphorylation like the wild type protein, although the timing appeared slightly different. Since p.T111I undoubtedly causes the CFNS phenotype, phosphorylation of Tyr324/329 may be less important for disease manifestation.

Reverse signalling influences actin cytoskeletal rearrangement and may result in transcriptional regulation of different genes involved in extracellular matrix reorganization (Campbell et al. 2008; Cowan and Henkemeyer 2001). NIH3T3 cells expressing either wild type or mutant ephrin-B1 exhibited differences in patches formation after EphB2-Fc stimulation. Cells expressing wild type or mutant ephrin-B1 were visualized by the green fluorescent protein and showed a scattered distribution in tissue culture dishes before stimulation. EphB2-Fc stimulation induced formation of patches in the wild type and p.T111I protein expressing cells, whereas no cell patches were found in p.P54L expressing cells. This suggests that phosphorylation of Tyr324/329 is closely linked with the patches formation, however, the mechanism of this linkage is unclear. Possible reasons are that wild type and p.T111I expressing cells experience a proliferative signal upon EphB2 stimulation. After division daughter cells do not move apart but rather stay close together. Alternatively, cells respond to EphB2 stimulation with increased motility and migrate into clusters. For ephrin-B2 it has been shown in the mouse that restriction of cell intermingling and communication was dependent on bi-directional signalling, whereas unidirectional activation of ephrin-B2 by recombinant truncated EphB2 was sufficient to restrict cell-cell communication through gap junctions (Mellitzer et al. 1999). Similar observations have been reported for ephrin-B1 by Davy et al. (2006) who proposed that regulation of gap junctions contributes to cell sorting downstream of Eph – ephrin interaction. In addition, embryonic mouse cells expressing an ephrin-B1 lacking the most C-terminal PDZ binding domain do not sort-out from wild type cells, whereas ephrin-B1 null cells do (Compagni et al. 2003; Davy et al. 2004, 2006). In this respect, it is striking that CFNS-causing missense mutations have been detected exclusively in the exons encoding the extracellular region of ephrin-B1, which strongly argues for the involvement of Eph receptor forward signalling in the pathogenic mechanism. We propose that CFNS is caused mostly by disturbance of Eph receptor forward signalling and the consequences of cellular interference in heterozygous females.

What is the role of ephrin-B1 expressing cells? After EphB/ephrin-B1 system activation EphB expressing cells begin to repulse (Mellitzer et al., 1999). We

proposed that ephrin-B1 cells stop their moving and attach themselves to the surface. This hypothesis is proven by patches formation analysis. Salvucci et al. (2006) have described an influence of ephrin-B1 on CXCR4 signalling in T-cells. This protein is important for cell migrations such as T-cells' homing (Busillo and Benovic, 2007). Ephrin-B1 activates PDZ-RGS3 that downregulates the CXCR4 activation preventing chemotaxis of the T-cells (Sharfe et al., 2002).

As it was told before, we assume that the major part of all PTC-causing mutations has neither dominant-negative, nor gain-of-function effects but rather loss of function effect. In this part of work we found out that p.P54L mutations also seemed to have a loss-of-function effect. According to our results and previously published data, we assume that the majority of disease-linked *EFNB1* mutations cause loss-of-function effect.

Analysis of the other Tyr residues (Tyr313, Tyr317, Tyr343 and Tyr344) phosphorylation may show whether p.P54L lacks the phosphorylation completely or in this case only the phosphorylation of Tyr324 and Tyr329 is disturbed. Analysis of the Tyr phosphorylation in wild type and p.T111I would reveal the crucial for the CFNS manifestation Tyr residues.

It is known that ephrin-B1 needs several other proteins such as Grb4 and Src kinases for the reverse signalling. These proteins form a complex with ephrin-B1 after it binds EphB2. It would be interesting to analyse the formation of this complex with the p.P54L and p.T111I ephrin-B1.

4.3. Real-time PCR

As it was said before, Bong et al. (2007) reported that Tyr329 is important for the ephrin-B1 – STAT3 complex formation. Hence, we expect disturbance of STAT3 signalling in p.P54L expressing cells. Stat3 is an important transcription factor, e.g. it controls expression of Twist1 (Cheng et al., 2008) and mutations of *Twist1* gene are involved in craniosynostosis formation (Merrill et al., 2006). It is also known that Msx2 works together with Twist1 in coronal suture formation.

The pathway of the ephrin-B1 reverse signalling is not clear till now. But it is known that *EFNB1* mutation may lead to the craniosynostosis formation. That is why we tried to find a link between ephrin-B1 pathway and STAT3 pathway.

Twist1 and *Msx2* expression experiments were performed in primary fibroblasts (not shown) and in transfected NIH 3T3 cells. In both cases real-time PCR did not indicate a change in the expression levels of these genes exceeding the 2 fold. While expression level of *Twist1* and *Msx2* do not change in response to ephrin-B1 stimulation, it is proposed that the Stat3 signalling involves some other proteins. In this case, there are other genes that are involved in ephrin-B1 reverse signalling. To answer this question, other genes from the *Twist1* pathway need to be analysed. Also it would be interesting to investigate pathways of the growth factors and cytokines related to neural stem cells.

Conclusions

1. In this work three PTC-causing mutations were analysed: frameshift c.377_384delTCAAGAAG, splice-site c.406+2T>C and deletion 614_615delCT. One mutation (c.377_384delTCAAGAAG) was shown to express no mutant transcript. Two other mutations give rise to the mutant RNA, but no mutant protein was detected. According to these results and previously published data, it can be concluded, that the majority of PTC-causing *EFNB1* mutations have neither dominant-negative, nor gain-of-function effects but rather loss of function effect.

2. The analysis of missense mutations c.161C>T/p.P54L and 332_333CC>TA/p.T111I revealed that both cause CFNS but have different mechanisms of ephrin-B1 signalling disturbance. Mutation p.P54L seems to have loss-of-function effect (no Tyr324/329 phosphorylation of the p.P54L ephrin-B1 and no patches formation of the p.P54L expressing cells were shown), whereas p.T111I ephrin-B1 differs slightly from the wild type in phosphorylation timing, and p.T111I-expressing cells show patches formation. Therefore, additional mechanisms involved in phenotypic manifestation need to be postulated. This may include either other tyrosine residues of ephrin-B1 are more important for reverse signalling. Another possibility could be impaired forward signalling of Eph receptor expressing cells. Combined with cellular interference this may be the main pathogenic mechanism in CFNS manifestation in female patients.

3. Expression of *Twist1* and *Msx2* genes was analysed in response to ephrin-B1 stimulation. Only the minor changes of the *Twist1* and *Msx2* expression level were found after the ephrin-B1 stimulation in wild type and in mutant ephrin-B1 expressing cells. From this we may conclude that *Twist1* and *Msx2* are not the main targets of the ephrin-B1 mediated STAT3 signalling.

Summary (English)

Ephrin-B1 protein forms signalling complexes with EphB receptor expressed in complementary cells. This complex was shown to work as a bi-directional signalling system, and ephrin-B1 was called “receptor-like protein”. Ephrin-B1 is encoded by *EFNB1* gene. Mutations of this gene cause the X-linked disease called craniofrontonasal syndrome (CFNS). This disease shows an unusual phenotypic pattern of inheritance, it affects heterozygous females more severely than hemizygous males although *EFNB1* is located on the X chromosome and has no homologue on the Y chromosome. This inheritance has been explained by random X-inactivation in heterozygous females and the consequences of cellular interference of wild type and mutant *EFNB1*-expressing cell populations with EphB-receptors expressing cells. Several patient derived *EFNB1* mutations have been analysed in this work, which consists of two parts. First one premature termination codon (PTC)-causing mutations were analysed: frameshift mutation c.377_384delTCAAGAAG, frameshift mutation c.614_615delCT (PTC in exon 4) and splice-site mutation c.406+2T>C (PTC in intron 2 or exon 3). All three mutations were predicted *in silico* to result in a truncated, soluble ephrin-B1 protein. Such protein would cause distant interaction with EphB-receptors. In the second part of this work the impact of missense mutations c.161C>T/p.P54L and c.332C>T/p.T111I on cell behaviour and reverse ephrin-B1 cell signalling was investigated. The role of these mutations was analysed in a cell culture model using NIH3T3 fibroblasts. This cell line was chosen because it does not express *Efnb1*, the mouse homologue of *EFNB1* gene. Both missense mutations are located in the extracellular ephrin domain that is involved in Eph-ephrin-B1 recognition and higher order complex formation. Reverse signalling of ephrin-B1 involves phosphorylation of several conserved tyrosine residues of the cytoplasmic tail. Two of them, Tyr324 and Tyr329 in human ephrin-B1, were shown to be the most important for signal transduction. To analyse the impact of missense mutations in ephrin-B1 signalling the phosphorylation of these two amino acids in mutant and wild type ephrin-B1 was monitored after the EphB2-Fc stimulation. It was reported previously, that ephrin-B1 is involved in STAT3 signalling pathway. Also, it is known that STAT3 controls the expression of *TWIST1* gene and *MSX2* is also involved in STAT3 signalling. According to that, the change of expression level of *MSX2* and *TWIST1* due to ephrin-B1 stimulation was monitored.

Mutation c. .377_384delTCAAGAAG showed a strong decrease of the mutant transcript level, mutations c.614_615delCT and c.406+2T>C showed the presence of the mutant RNA but not the mutant protein.

Mutation p.P54L showed no phosphorylation of the Tyr324/329, wild type and p.T111I mutation showed the difference in the phosphorylation timing. Also wild type and p.T111I expressing cells showed patches formation after the EphB2-Fc stimulation whereas p.P54L expressing cells remained to be scattered.

MSX2 and *TWIST1* genes showed only a minor change of the transcription level in response to the ephrin-B1 stimulation with EphB2.

Four from the five investigated *EFMB1* mutations appeared to have rather loss-of-function.

Zusammenfassung (Deutsch)

Das Protein Ephrin-B1 bildet einen Signalkomplex mit dem EphB-Rezeptor, der in komplementären Zellen exprimiert wird. Es wurde gezeigt, dass dieser Komplex als ein bidirektionales Signalsystem funktioniert und deswegen wurde Ephrin-B1 als Rezeptor-ähnliches Protein benannt. Ephrin-B1 wird vom *EFNB1* Gen kodiert. Mutationen des *EFNB1*-Gens verursachen eine X-chromosomale Erkrankung – das Craniofrontonasale Syndrom (CFNS). Diese Erkrankung zeigt ein ungewöhnliches Vererbungsmuster: heterozygote Frauen weisen das Vollbild der Erkrankung auf, während hemizygote Männer keine oder nur eine leichte Symptomatik zeigen, obwohl das *EFNB1* Gen auf dem X-Chromosom lokalisiert ist und kein homologes Gen auf dem Y-Chromosom hat. Dieses Vererbungsmuster wurde durch zufällige X-Inaktivierung bei heterozygoten Frauen und als Folgen der zellulären Interferenz erklärt. Die zelluläre Interferenz entsteht durch Ergebnis von mutierten und Wildtypzellen mit EphB-Rezeptor expremierenden Zellen. In dieser Arbeit wurden einige *EFNB1* Mutationen die bei Patienten identifiziert werden, untersucht. Die Arbeit besteht aus zwei Teilen. Der erste Teil beinhaltet die Analyse von drei Mutationen, die eine vorzeitige Termination (PTC, premature termination codon) verursachen: Leseraster Mutation c.614_615de1CT (PTC in Exon 4) und Spleiß-Mutation c.406+2T>C (PTC in Intron 2 oder Exon 3). Es wurde vorhergesagt, dass die Anwesenheit jeder dieser Mutationen zur Bildung eine verkürzten, löslichen Form des Ephrin-B1 Proteins führt. Dieses verkürzte Protein könnte eine Blockierung des EphB-Rezeptors bewirken. Der zweite Teil der Arbeit beinhaltet die Untersuchung der Missense Mutationen c.161C>T/p.P54L und c.332C>T/p.T111I auf das Zellenverhalten und die Ephrin-B1 Signaltransduktion. Die Auswirkungen dieser Mutationen wurde im Zellkulturmodell unter Anwendung von NIH3T3 Fibroblasten analysiert. Diese Zelllinie wurde ausgewählt, weil sie kein Ephrin-B1 der Maus exprimiert. Beide Missense Mutationen sind in der extrazellulären Ephrin-Domäne lokalisiert. Diese Domäne kann zur Erkennung von Eph-Rezeptor und Ephrin-B1 und zur Bildung eines hoch-komplexen Multiproteins beitragen. Bei der Durchführung des reversen Ephrin-B1 Signals ist die Phosphorylation der bestimmtes evolutionär konservierten Tyrosin-Reste des zytoplasmatischen Anteiles notwendig. Es wurde gezeigt, dass die beiden Tyr324 und Tyr329 im menschlichen Ephrin-B1 Protein besonders wichtig für die Signaltransduktion sind. Um zu analysieren, welche Rolle Missense Mutationen bei der Ephrin-B1 Signaltransduktion spielen, untersuchte ich

die Phosphorylierung dieser zwei Aminosäuren bei Mutanten und Wildtyp Ephrin-B1 nach der EphB2-Fc Stimulation. Frühere Ergebnisse zeigten, dass Ephrin-B1 Protein den STAT3 Signalwege aktiviert. Es ist auch bekannt, dass STAT3 die Expression des *TWIST1* Gens kontrolliert und das *MSX2* Gen in den STAT3 Signalwege integriert ist. Aus diesem Grund wurde Expression des *MSX2* Gen und *TWIST1* Gens in Antwort auf die Ephrin-B1 Stimulation untersucht. Jedoch konnte keine Veränderung in der Expression dieses Gen festgestellt werden.

Es wurde gezeigt:

1. Mutation c.377_384delTCAAGAAG weist eine starke Reduzierung der Expression des mutant Allels auf. Mutationen c.614_615delCT und c.406+2T>C zeigten die Expression des mutant Allels, aber kein mutant Protein war zufinden.
2. Mutation p.P54L weist keine Tyr324/329 Phosphorylation auf, Wildtyp und p.T111I Mutation demonstrierten nur geringe Differenzen zwischen den Phosphorylation Zeit.
3. MSX2 und TWIST1 Gene zeigten geringe Differenzen in der Änderung der Expression in Antwort auf die Ephrin-B1 Stimulation.

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Abbreviations

aPKC – atypical protein kinase C

CFNS – craniofrontonasal syndrome

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

FCS – fetal calf serum

FGF – fibroblast growth factor

Grb – growth-factor-receptor-bound protein

GRIP – glutamate receptor–interacting protein

HUMARA – human androgen receptor assay

LTP – long terminus potential

miRNA – micro RNA

MMP-8 – matrix metalloprotease-8

mRNA – matrix RNA

NMD – nonsense-mediated mRNA decay

PB1 domain – Phox and Bem 1 domain

PDZ domain – post synaptic density-95, disclarge and zonulin-1 proteins domain

PTC – premature termination codon

RTK – receptor tyrosine kinase

PTP – phosphotyrosine phosphatase

RNA – ribonucleic acid

SFK – Src family tyrosine kinase

SNP – single nucleotide polymorphism

SH domain – Src homology domain

Tyr – tyrosine

VEGF – vascular endothelial growth factor

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Makarov R.P., Feoktistova M.A., Hartina G.A. Gene analysis of fasciations character of Pea (*Pisum sativum* L.). „Actual problems of genetic“, Materials of 2nd conference of Moscow Vavilov N.I., society of genetics and selectionists. Moscow, Russia, 20-21 of February 2003. V.1, p. 145; M. 2003. MACA, (Russian). The author received the poster award for this poster.

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Makarov R., Steiner B., Preisler-Adams S., Rauch A., Wieacker P., Wieland I. Expression and functional analysis of *EFNB1* mutations in craniofrontonasal syndrome. Poster at the European Human Genetics Conference of the European Society of Human Genetics. Vienna, Austria, 23-26 May 2009 (English). The author received the poster award for this poster.

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Wieland I., Makarov R., Reardon W., Tinschert S., Goldenberg A., Thierry P., Wieacker P. (2008) Dissecting the molecular mechanisms in craniofrontonasal syndrome: differential mRNA expression of mutant *EFNB1* and the cellular mosaic. European Journal of Human Genetics 6: 184-191.

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