Bone morphogenetic proteins such as BMP4 are essential for proper development of telencephalic forebrain structures and induce differentiation of telencephalic neural stem cells into a variety of cellular fates, including astrocytic, neuronal, and mesenchymal cells. Little is yet understood regarding the mechanisms that underlie the spatiotemporal differences in progenitor response to BMP4. In a screen designed to identify novel targets of BMP4 signaling in telencephalic neural stem cells, we found the mRNA levels of the previously uncharacterized factor CXXC5 reproducibly up-regulated upon BMP4 stimulation. In vivo, CXXC5 expression overlapped with BMP4 adjacent to Wnt3a expression in the dorsal regions of the telencephalon, including the developing choroid plexus. CXXC5 showed partial homology with Idax, a related protein previously shown to interact with the Wnt-signaling intermediate Dishevelled (Dvl). Indeed CXXC5 and Dvl co-localized in the cytoplasm and interacted in co-immunoprecipitation experiments. Moreover, fluorescence resonance energy transfer (FRET) experiments verified that CXXC5 and Dvl2 were located in close spatial proximity in neural stem cells. Studies of the functional role of CXXC5 revealed that overexpression of CXXC5 or exposure to BMP4 repressed the levels of the canonical Wnt signaling target Axin2, and CXXC5 attenuated Wnt3a-mediated increase in TOPFlash reporter activity. Accordingly, RNA interference of CXXC5 attenuated the BMP4-mediated decrease in Axin2 levels and facilitated the response to Wnt3a in neural stem cells. We propose that CXXC5 is acting as a BMP4–induced inhibitor of Wnt signaling in neural stem cells.

Members of the TGFβ family such as bone morphogenetic proteins (BMP) influence multiple essential events during brain development, such as differentiation, proliferation, and migration (1–3). Stimulation of telencephalic neural stem cells by BMP4 induces differentiation into a variety of cellular fates, including neuronal, astrocytic and smooth muscle cells in vitro, and genetic studies have shown that BMP4 is essential for proper differentiation and regionalization of the telencephalic forebrain (1, 2, 4, 5). BMP4 mediates its effects through nuclear translocalization of Smad proteins such as Smad1 and Smad4 that can act directly as transcription factors and associate with a number of important cofactors, including TGF, Sip1, and CBP/p300 (6, 7).

BMP activity exert cross-talk with many signaling pathways, such as the membrane-bound receptor Notch, fibroblast growth factors (FGFs), and Wnt factors (8, 9), and it has been proposed that BMP molecules act in synergy with canonical Wnt signaling molecules, such as Wnt3a, to regulate telencephalic regionalization (1, 10). Less is known regarding downstream targets of BMP signaling that regulate the spatial and temporal context-specific differences in progenitor responsiveness to extracellular signaling factors.

BMP activity is directly regulated by extracellular inhibitors such as noggin and chordin, and Wnt signaling activity is regulated at many levels by both extra- and intracellular mechanisms. An example of an intracellular inhibitor of Wnt signaling with reported effects on forebrain development is Idax. Idax binds directly to the Wnt signaling mediator Dishevelled (Dvl) and seems to compete at the binding site of Axin, resulting in an inhibition of Wnt activity (11). Idax belongs to the CXXC family of proteins of which other members, such as MBD1, are predominantly nuclear DNA-binding proteins with chromatin-modifying properties (12). Dvl is predominantly albeit not exclusively localized in the cytoplasm, and Idax appears to be an exception to other CXXC domain-containing proteins, being localized largely in the cytoplasm (11). It has been shown that some CXXC proteins are cancer-associated genes that can regulate the activity and function of important transcriptional complexes, such as Polycomb proteins (12). Idax is expressed in...
In a systematic attempt to increase the understanding of the mechanisms underlying cell context-specific responses of telencephalic neural stem cells (NSCs) to BMP4 signaling during forebrain development, we have pursued gene expression profiling analysis using microarrays to identify putative direct and novel targets for BMP4 signaling in NSCs. By this approach, we identified CXXC5 as a direct BMP4 target in NSCs. In situ hybridization experiments revealed that CXXC5 expression localized selectively to the BMP4-rich dorsal telencephalon adjacent to the neighboring regions expressing Wnt3a. Although CXXC5 displayed predominantly nuclear localization, it co-localized and interacted with several Dvl isoforms in the cytoplasm. CXXC5 influenced the expression of the endogenous Wnt signaling target Axin2 as well as Wnt3a-induced activation of a TOPflash reporter. Based on these results, we propose that CXXC5 is a novel BMP-regulated modulator of Wnt signaling and targets thereof in telencephalic NSCs.

**EXPERIMENTAL PROCEDURES**

**Cortical NSC Cultures**—NSCs were obtained from the dissociated cerebral cortices of Sprague-Dawley rat E15.5 embryos and cultured as previously described (14–16). Briefly NSCs were cultured in serum-free DMEM:F12 media (Invitrogen) enriched with N2 supplement and grown on poly-l-ornithine/fibronectin (Sigma)-coated cell culture dishes (Corning). Cells were maintained in a proliferative state using 10 ng/ml FGF2 (R&D Systems) and passed once before nucleofections or twice before stimulations. After the second passage, cells were plated at 500–10,000 cells/cm² and allowed to proliferate for twice before stimulations. After the second passage, cells were transfected using nucleofection (Amaxa, Germany) and passaged once before nucleofections or fibronectin (Sigma)-coated cell culture dishes (Corning). Cells changed every 48 h.

For inhibition of protein synthesis, 10–20 μg/ml cycloheximide (Sigma) were added to the media with or without the addition of other soluble factors. BMP4 and Wnt3a (R&D Systems) were both used at 10 ng/ml. Addition of soluble factors was carried out every 24 h, and media was changed every 48 h. For inhibition of protein synthesis, 10–20 μg/ml cycloheximide (Sigma) was added to the media 15–30 min before addition of BMP4. All experiments in this study involving animals and stem cell isolation were approved by the ethics committee for animal research in Stockholm, Sweden.

**Gene Expression Profiling Using Microarrays**—Rat neural stem cells were exposed to BMP4 or kept in FGF2 for 3 h before the cells were lysed and RNA extracted (Qiagen). RNA from the time for stimulation start was extracted and used as control. The microarray was carried out by KI seq-express (former KIChip). Briefly, RNA was amplified (17) and labeled with Cy3 (PerkinElmer) according to the supplier’s instructions. The BMP4 and Wnt3a constructs were kind gifts from Dr. John L. R. Rubenstein.

**In Vitro**—Non-radioactive in situ hybridization on sections was performed as previously described (20). Heads (E10.5, E12.5, and E14.5) of mouse embryos were fixed in 4% paraformaldehyde (Sigma) for 2–5 h and then transferred to 30% sucrose at 4 °C overnight before frozen in Tissue-Tek (Sakura Finetek) on dry ice and kept in −70 °C until cryostat-sectioned (Microm) onto SuperFrost Plus glasses (Menzel-Gmbh & Co KG) as 12-μm sections. Prior to hybridization, sections were permeabilized with 1 μg/ml proteinase K (Invitrogen), acetylated with 13.5 ml/L triethanolamine (Sigma), 44 mM acetic anhydride (Sigma) and 1.7 mM HCl (Merck), and pre-hybridized with hybridization buffer (50% formamide (Fluka), 5× SSC, 5× Denhardt’s solution (Sigma), 250 μg/ml bakers’ yeast RNA (Sigma), 500 μg/ml herring sperm DNA (Invitrogen), 1 g/50 ml blocking reagent (Boehringer)) for 3–6 h. Hybridization was carried out at 70 °C overnight with 1–4 μl of DIG-labeled probe (0.1–0.2 μg/μl) per 100 μl of hybridization buffer and glass. Post-hybridization washes were performed for 1–2 h at 70 °C in 0.2× SSC. Sections were then blocked with 10% fetal calf serum for 2–4 h before being subjected to an anti-DIG antibody (1:5000, Roche) at 4 °C overnight. Finally, the slides were submerged into a 10% polyvinyl alcohol (Sigma) solution containing 1.72 ml/l NBT (Roche) and 1.72 ml/l B-CIP (Roche) chromogen components until sufficiently stained (3–72 h). DIG probes were produced according to the manufacturers’ instructions using DIG RNA labeling mix (Roche). After in situ hybridization, the sections were analyzed and micrographs obtained with a Zeiss Axioskop 2 mot plus microscope. CXXC5, BMP4, and Wnt3a probes were used on >3 littermate brains per developmental stage.
CXXC5 Is a Neural BMP4-induced Modulator of Wnt Signaling

Immunoprecipitation, Immunoblotting, and Immunocytochemistry—COS7 and HEK-293 cells were transfected with GFP-CXXC5, CXXC5-GFP, or vector control only containing GFP individually or cotransfected with a full-length XDi-shaved-Myc (21) using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Immunoprecipitation, immunoblotting, and culturing of COS7 and HEK-293 cells were performed essentially as previously described (22). Antibodies used for immunoprecipitation were anti-GFP (Fitzgerald Industries), rabbit anti-Myc (SCBT) and anti-Dvl3 (Cell Signaling Technology). Primary antibodies used for immunoblotting: anti-ß-catenin (BD Biosciences), anti-Dvl1 (SCBT), anti-Dvl2 (SCBT), anti-Dvl3 (SCBT), anti-GFP (Chemicon), mouse anti-Myc (SCBT), and anti-ß-actin (Abcam) (22). For immunocytochemistry, cultures were fixed using 10% formalin (Sigma) 24 h after transfections. Immunostaining was performed using standard protocols using chicken polyclonal anti-GFP (Chemicon, 1:1000) and mouse monoclonal anti-c-Myc (SCBT, 1:200) followed by appropriate species-specific Alexa-488 and Alexa-594-conjugated secondary antibodies (Molecular Probes, 1:500). Nuclei were visualized using Vectashield containing DAPI (Vector Laboratories, Inc.). Fluorescent and brightfield images were acquired using Zeiss Axioskop2/MRm camera with Axiovision software. Images were assembled using Adobe Photoshop.

FRET—To perform Förster (Fluorescent) Resonance Energy Transfer (FRET) measurements, we used a Zeiss LSM 5 Exciter inverted confocal scanning laser microscope using a ×63/1.2 NA objective as described previously (23). CXXC5-GFP-expressing NSCs were grown on prepared glass coverslips, and 24 h after nucleofection, cells were fixed using a 50% methanol/50% acetone fixing solution and then processed for immunocytochemistry. The coverslips were mounted on slides using the ProLong Antifade mounting media kit (Molecular Probes, Invitrogen) to enable perfect conservation of the fluorescent signal. A detailed description of the FRET technique can be found elsewhere (24, 25). The Förster constant, R₀, for the donor-acceptor pair, GFP and Alexa-555, used in this study was 6.3 nm (Handbook of Probes, Invitrogen). FRET occurs when the fluorophores are separated by distances 0.5 R₀ < r < 2 R₀. Thus, it is possible to distinguish proteins that are spatially colocalized within a ~14-nm radius. To determine FRET, we quantified the quenching of donor fluorescence by performing acceptor photobleaching. NSCs expressing CXXC5-GFP and stained with Alexa555-labeled secondary goat anti-rabbit IgG antibody (Molecular Probes, Invitrogen; 1:100) to detect rabbit polyclonal antibody to Dvl2 (SCBT; 1:100) were excited with the 488 and 561 nm lasers. The acceptor, Alexa555, was then irreversibly photobleached in a selected adequate cytoplasmic region (~5–10 μm²) by continuous excitation with the 561 nm laser for 10–20 s. Thereafter, the residual Alexa555 and GFP image was obtained, and a region of interest (ROI) was outlined in the photobleached area and processed using Zeiss LSM image Examiner. Ratios between GFP intensities in the ROI, before and after photobleaching, were calculated to quantify FRET. The FRET values presented are corrected for erroneous intensity changes for each cell in two cytoplasmic ROIs randomly selected outside the bleached area. 10–25 cells were measured for each experiment.

Overexpression and siRNA-mediated Knockdown—NSCs were nucleofected according to the manufacturer’s instructions using program A–33 on the Nucleofector (Amaxa) as previously described (26). Experiments were commenced 24 h after nucleofection, during which NSCs were maintained in FGF2. As the healthiness of the cells varied significantly when obtaining very high (>|%90%) or very low (<20%) knockdown of CXXC5 expression levels, experiments where a knockdown of CXXC5 mRNA showed between 40–70% efficiency (n = 5) were chosen for detailed analysis. Transient transfection studies for luciferase assay in fibroblasts were performed essentially as previously described (27). Cells were plated in 24-well plates 24 h before transfection and transfected with 0.6 μg of plasmid DNA/well complexed with 2 μL of Lipofectamine 2000 (Invitrogen). Typically, cells were transfected with 200 ng of the reporter construct and increasing doses of CXXC5 expression vector. After 5 h of incubation, the lipid/DNA mix was replaced with fresh 10% serum medium. Cells were serum-starved 24 h post-transfection and stimulated with either vehicle or purified Wnt3a (R&D Systems). Luciferase activities were assayed using Dual-Luciferase Reporter Assay System (Promega), following the manufacturer’s protocol. Primary antibodies used for immunoblotting: anti-ß-catenin (BD Biosciences), anti-activated (dephosphorylated) ß-catenin (Upstate), anti-Dvl2 (SCBT), and anti-ß-actin (Abcam).

Statistical Analysis—Statistical analysis was performed in Prism4 (GraphPad software). Initially variance analysis (ANOVA) was performed, followed by Student’s t test (unpaired). Significance were assumed at the level of p < 0.05 (*, p < 0.05; ***, p < 0.001, see figure legends).

RESULTS

BMP4 Rapidly Induces Significant Changes in Gene Expression in Neural Stem Cells—To elucidate novel downstream targets of BMP signaling in neural progenitors, we used a well-characterized rat embryonic telencephalic NSC preparation that has previously been used to study BMP signaling. During the continuous administration of FGF2 the NSCs remain undifferentiated with the capacity to differentiate into neuronal, astrocytic, oligodendrocyte, and mesenchymal cells (14–16, 26). Upon BMP4 stimulation these cells respond with increased Smad phosphorylation, subsequent nuclear translocation of Smads and increase in transcription of Smad target genes (5), and subsequent differentiation into astrocytic and mesenchymal cells (5, 28).

After primary FGF2 expansion, one passage and continued FGF2 expansion, NSCs were treated with either FGF2 or BMP4 for 3 h. This time point was chosen due to the rapid induction (~30 min) of Smad-mediated transcription downstream of BMP4 stimulation (5). The gene expression profile was subsequently investigated using microarrays. This procedure identified 162 spots that were putatively (p value < 0.05) up- or down-regulated more than 1.5-fold after 3 h (supplemental data). To initially validate the array, we picked ~10% of the identified genes and performed quantitative RT-PCR (RT-qPCR) on the same samples that were used for the microarrays. This experiment showed that around 90% of the genes identified in the
screen were up- or down-regulated, in approximate accordance with the chosen $p$ value predicting 95% accuracy (see further below).

The effects of BMP4 stimulation of NSCs are highly dependent on the microenvironment and cell culture conditions, such as plating density (4, 28). To analyze the reproducibility of the effects of BMP4 on the expression of the identified genes in NSCs, we therefore picked eight factors from our screen (Fig. 1A and see below), including known and potentially novel targets of BMP4, and repeated the experimental procedure four times. Six out of these eight mRNAs were reproducibly BMP4-regulated in NSCs: Gas1, Olig1, Id2, Idb4, CXXC5, and Hey1 (Fig. 1A), whereas two mRNAs, Sin3A and Skiip, were regulated only in occasional experiments (data not shown).

**CXXC5 Is a Novel Direct Target of BMP4 in NSCs**

—Id2 and Idb4 have previously been shown to be direct targets of BMP signaling. These factors interact with and down-regulate the activity of bHLH proteins, such as the oligodendrocyte-associated bHLH transcription factors Olig1 and Olig2 thus interfering with oligodendrocyte differentiation (29). In addition to this mechanism, we found that Olig1 expression was significantly down-regulated by BMP4 stimulation (Fig. 1A), suggesting that BMP4-mediated inhibition of oligodendrocyte differentiation may occur at several levels. Other reproducibly BMP4-regulated mRNAs were the Notch-associated repressor Hey1 (8), and Gas1 recently linked to Sonic Hedgehog signaling (30–32) (Fig. 1A).

Another significantly BMP4-regulated mRNA was the previously uncharacterized factor CXXC5 (Fig. 1A). CXXC5 expression levels started to increase at 40 min, were high at 60 min and remained up-regulated at 10 h after BMP4 exposure (see further below and data not shown).

**FIGURE 1.** BMP4 exposure results in rapid changes in gene expression in telencephalic NSCs. **A**, levels of mRNA expression relative to control mRNA (HPRT) of six putative target genes (Gas1, Olig1, Id2, Idb4, CXXC5, Hey1) in NSCs after BMP4 treatment and relative to unstimulated control as assessed by RT-qPCR. **B**, RT-qPCR assessment of the relative CXXC5 mRNA expression levels to HPRT after BMP4 stimulation with or without pretreatment with the protein synthesis inhibitor cycloheximide (CHX) compared with unstimulated control.

**FIGURE 2.** CXXC5 mRNA is expressed at high levels in the dorsal telencephalon, the expression overlaps with BMP4 and is adjacent to Wnt3a expression. **A–I**, micrographs depicting in situ hybridization results of transverse sections of mouse brains at embryonic ages (E) 10.5 (**A**–**C**), 12.5 (**D**–**F**), and 14.5 (**G**–**I**) hybridized with probes for detection of CXXC5 (**A**, **D**, **G**), BMP4 (**B**, **E**, **H**), and Wnt3a (**C**, **F**, **I**). At E10.5, the expression patterns overlap significantly. At E12.5, CXXC5 and BMP4 mRNA expressions overlap significantly and are detected in the very close vicinity of Wnt3a labeling. At E14.5, very low levels of CXXC5 transcripts were detected, whereas BMP4 mRNA was found in and around the developing choroid plexus, and Wnt3a labeling was detected just lateral to the BMP4-expressing domain. Arrows mark regions of high specific labeling.
CXXC5 Is a Neural BMP4-induced Modulator of Wnt Signaling

To investigate whether CXXC5 could be a direct target of BMP4 signaling, we pretreated the NSCs with cycloheximide (CHX), an inhibitor of translation and thus de novo protein synthesis. We then evaluated the expression levels of CXXC5 3 h after subsequent FGF2 versus BMP4 stimulation. Pretreatment with CHX 15–30 min before BMP4 or FGF2 stimulation did not significantly inhibit BMP4-mediated up-regulation of CXXC5 (Fig. 1B), suggesting that the increased levels of CXXC5 mRNA could be the result of a direct effect of BMP4 signaling on CXXC5 gene expression.

CXXC5 Is Highly Expressed in the Dorsal Telencephalon Where BMP4 Expression Is Enriched—To confirm the gene product of CXXC5, we performed in vitro transcription/translation by TNT using a sequenced construct harboring full-length CXXC5. The protein product revealed a major band migrating at around 38 kDa (data not shown), in close range of the predicted protein product of the CXXC5 gene.

To assess the gene expression pattern of CXXC5 in vivo in relation to BMP4 in neural progenitors, we performed in situ hybridization on forebrain sections from mice at embryonic age (E) 10.5, 12.5, and 14.5. At E10.5 and E12.5, CXXC5 expression was largely restricted to the dorsal pallium (Fig. 2, A and D). These regions are known to be rich in expression of BMP receptors as well as several BMP molecules, including BMP4 (2, 3). Indeed in situ hybridization experiments using a probe for BMP4 demonstrated a clear overlap in expression of CXXC5 and BMP4 in the dorsal pallium (Fig. 2, A, B, D, and E). Importantly, CXXC5 expression was prominent in and around the developing choroid plexus (Fig. 2D), a non-neuronal structure producing and secreting the cerebrospinal fluid. At E14.5 however, only low levels of CXXC5 expression were detected (Fig. 2G), whereas BMP4 transcripts displayed a high expression selectively in the ventricular zone at the region of the developing choroid plexus (Fig. 2H).

In addition to BMP4, the signaling molecule Wnt3a has been shown to be required for proper development of the dorsal telencephalon, influencing the development of the hippocampal formation and pyramidal neurons therein (10). At E10.5, Wnt3a mRNA showed an overlapping expression with CXXC5 and BMP4 (Fig. 2, A–C), while at E12.5 Wnt3a transcripts showed a marked reduction in expression just at the border of CXXC5 and BMP4 expression (Fig. 2, D–F), possibly demarcating the regions of the developing hippocampal formation and the choroid plexus. At E14.5 a marked expression of the Wnt3a gene was detected just lateral to the BMP4 expression (Fig. 2, H and I). The expression pattern of CXXC5 thus suggests that this factor could play a role in telencephalic development and strengthens the association between CXXC5 and high BMP signaling activity.

CXXC5 Interacts with the Wnt Signaling Mediator Dishevelled in Co-immunoprecipitation Experiments—CXXC5 has been predicted to be a parologue of another CXXC domain containing factor named Idax (CXXC4) (11, 12, 33). Idax was identified in a screen for proteins interacting with the PDZ domain of the intracellular signaling factor Dishevelled (Dvl). Dvl is a predominantly cytoplasmic protein (34, 35) and acts as an intermediate of canonical Wnt signaling. Accordingly, Idax was found to inhibit canonical Wnt signaling as assessed by transcriptional assays (11). A model of the sequence in the domain of Idax that interacts with Dvl has been generated and this sequence shows 100% similarity to a sequence in CXXC5 (supplemental data). We, therefore, generated constructs where GFP was fused to the N or C terminus of CXXC5 to investigate whether Dvl interacts with CXXC5 in a similar manner as Idax. After co-transfection of the GFP fusion proteins with full-length Myc-Dvl in COS7 and HEK-293 cells, we immunoprecipitated whole cell protein extracts using Myc- or
Transfection of the GFP-CXXC5 construct into COS7 cells investigated the subcellular localization of CXXC5 and Dvl. CXXC5 and Dvl occurred in the nucleus or in the cytoplasm, we localization (34). To investigate whether the interaction of motifs. It has further been reported that Dvl can display nuclear nucleus, despite the lack of classical nuclear localization signal protein, though Idax has been demonstrated to be largely a cytoplasmic localization and Co-localizes with Dvl in the Cytoplasm

CXXC5 Displays Both Nuclear and Cytoplasmic Subcellular Localization and Co-localizes with Dvl in the Cytoplasm—Although Idax has been demonstrated to be largely a cytoplasmic protein, in silico screens predicted CXXC5 to localize to the nucleus, despite the lack of classical nuclear localization signal motifs. It has further been reported that Dvl can display nuclear localization (34). To investigate whether the interaction of CXXC5 and Dvl occurred in the nucleus or in the cytoplasm, we investigated the subcellular localization of CXXC5 and Dvl. Transfection of the GFP-CXXC5 construct into COS7 cells revealed that CXXC5 located both to the nucleus and the cytoplasm (Fig. 4A). Nuclear GFP-CXXC5 could be detected in 90–95% of the transfected cells whereas clear cytoplasmic labeling was detected in ~30–40% of the transfected cells. The nuclear labeling was homogeneous and associated with DNA as assessed by merging with the DAPI staining (Fig. 4D). The cytoplasmic labeling of CXXC5 displayed characteristics of large bodies in close proximity to the nucleus, localized just outside the nuclear membrane. Transfections of Myc-Dvl revealed a predominantly cytoplasmic localization (Fig. 4B). Co-transfection revealed that CXXC5 and Dvl indeed co-localized extensively in the cytoplasm (Fig. 4, C and D). The results showed some experimental variations but yielded an average of co-localization in around 40% of the co-transfected cells.

**FRET Experiments Show that CXXC5-GFP and Dvl2 Are Located in Close Proximity in Neural Stem Cells**—All proteins of the Dishevelled family are expressed during the development of the nervous system, and Dvl2 is highly expressed in forebrain progenitors. In order to investigate the interaction between endogenous Dvl2 and CXXC5 specifically in NSCs, we performed Förster FRET experiments (23–25). For FRET quantification we used the acceptor photobleaching method on NSCs nucleoected with CXXC5-GFP. Endogenous Dvl2 was detected in NSCs by immunocytochemistry using polyclonal anti-Dvl2 IgG. CXXC5-GFP served as FRET donor. The primary antibody against Dvl2 was probed with an Alexa555-conjugated IgG secondary antibody, which served as the FRET acceptor (Dvl2-Alexa555). In each cell, we measured the intensity of the CXXC5-GFP and Dvl2-Alexa555 in a subcellular ROI showing overlap of the two proteins, before and after irreversible and total acceptor photobleaching (Fig. 5A). Subsequently, two subcellular regions were randomly selected away from the photobleached region and the intensity of CXXC5-GFP was measured before and after photobleaching. These values were used to correct the value measured in the bleached region for erroneous intensity changes.

Following acceptor photobleaching, the CXXC5-GFP fluorescence intensity in the ROI was significantly enhanced to 108.0 ± 0.7% of the intensity before photobleaching (p < 0.001, n = 23, Fig. 5B). These results implied that the donor and acceptor complexes, CXXC5-GFP and Dvl2-Alexa555, were in close proximity, separated by less than 14 nm, i.e. the maximal distance for FRET detection between GFP and Alexa555 (24).
CXXC5 Is a Neural BMP4-induced Modulator of Wnt Signaling

To ensure that the resulting increase in CXXC5-GFP fluorescence intensity was a specific consequence of the photobleaching of Dvl2-Alexa555 and not a result of laser excitation, we decreased the laser power during photobleaching to levels unable to fully bleach Dvl2-Alexa555. Dvl2-Alexa555 fluorescence intensity following the photobleaching protocol was reduced to 20–30% (20% laser power), 40–50% (10% laser power), or unchanged (0.1% laser power). In none of these cases was the CXXC5-GFP fluorescence intensity enhanced after photobleaching (20% laser power, 101.2 ± 1.3, n = 7; 10% laser power, 97.8 ± 3.3, n = 7; 0.1% laser power, 98.8 ± 0.6, n = 6).

To further assure that the observed FRET between CXXC5 and Dvl2 uniquely reflected a property of this pair of proteins, we performed control experiments in NSCs using nucleofection and expression of either GFP alone or of another GFP-tagged cytoplasmic protein, NFATc1. FRET analysis was performed using GFP/GFP-NFATc1 (donor) and the same Alexa555-labeled secondary antibody to detect the Dvl2 antibody (Dvl2-Alexa555, acceptor). No significant change in donor emission ratio before and after acceptor photobleaching was found for neither of these molecular pairs (n = 11, data not shown).

Taken together, these results indicate that CXXC5 and Dvl2 are located in close proximity (<14 nm) strengthening the suggestion that there is a specific physical association between CXXC5 and Dvl2 in NSCs.

BMP4 Stimulation or Overexpression of CXXC5 Attenuates the Expression Levels of the Wnt3a Target Gene Axin2 in NSCs—To elucidate a functional role for the BMP4-induced increase in CXXC5 expression levels and the interaction between CXXC5 and Dvl, we next aimed at investigating the effects of CXXC5 overexpression on the response to Wnt signaling in the NSCs. In the developing nervous system, canonical Wnt signaling by Wnt3a has been shown to exert cross-talk with BMP signaling in the neural crest and in the dorsal regions of the developing telencephalon (1, 9, 10, 36) where high levels of CXXC5 expression were found (Fig. 2, A and D).

To first investigate the responsiveness of telencephalic NSCs to dorsally expressed signaling factors, we used RT-qPCR to estimate the endogenous levels of the bona fide Wnt target Axin2 in response to short-term exposure to recombinant BMP4 or Wnt3a. Axin2 gene expression is directly regulated by the Wnt signaling mediator β-catenin and is commonly used as a measurement for canonical Wnt signaling activity (37–39). BMP4 treatment led to significantly reduced levels of Axin2 expression within 6 h, in accordance with an up-regulation of CXXC5 and thus inhibitory effect on Wnt signaling activity (Fig. 6A). In contrast, Wnt3a stimulation resulted in a significant increase (≈4-fold) in Axin2 mRNA expression 4 h after stimulation compared with control (Fig. 6B, black bars).

To study whether the Wnt3a response would be affected by increased levels of CXXC5 as assessed by Axin2 expression levels, we next overexpressed CXXC5 in the NSCs by nucleofection. Nucleofection efficiency was estimated to vary between 30–60% in individual experiments with the NSCs. Overexpression of CXXC5 resulted in a marked attenuation of the Wnt3a-mediated increase in Axin2 expression levels (Fig. 6B). A decrease in Axin2 expression levels was seen also in FGF2-treated control cultures by CXXC5 overexpression (Fig. 6B). These results suggest that high levels of CXXC5 interfere with the response to Wnt3a stimulation in NSCs.

CXXC5 Overexpression Attenuates Wnt3a-mediated Increase in TOPflash Reporter Activity—To see whether the effect of CXXC5 on Wnt signaling was selective for Axin2 expression in NSCs or a general effect on canonical Wnt signaling, we next investigated the effects of CXXC5 expression on Wnt3a mediated activation of a TOPflash reporter in HEK-293 cells, a com-

![FIGURE 5. FRET experiments reveal that CXXC5-GFP and endogenous Dvl2 are localized in close proximity in neural stem cells.](image)

![FIGURE 6. BMP4 exposure or CXXC5 overexpression strongly reduces the expression of the canonical Wnt signaling target Axin2 in neural stem cells.](image)
monly used assay for studies of canonical Wnt signaling (11). Control experiments confirmed that reporter activity was robustly activated by overexpression of the canonical Wnt signaling mediator β-catenin (Fig. 7A). Transfections of increasing amounts of CXXC5 revealed that 100 ng of CXXC5 significantly inhibited the TOPflash reporter activity induced by 50 or 100 ng of recombinant Wnt3a (Fig. 7B). In addition, a decrease in Wnt3a-induced activated (dephosphorylated) β-catenin was observed in CXXC5-transfected cells, although no significant changes in the total protein levels of β-catenin or Dvl could be detected (Fig. 7, C and D). Hence these experiments strengthen the suggestion of a role for CXXC5 in modulating canonical Wnt signaling activity.

siRNA Against CXXC5 Attenuates the BMP4 Response and Facilitates the Wnt3a Response in NSCs—The results from the overexpression experiments allowed us to speculate whether a reduction of the endogenous levels of CXXC5 should attenuate the BMP4-mediated decrease in Axin2 expression levels and facilitate Wnt3a signaling effects in the NSCs. To study the role for CXXC5 in more physiological cellular conditions, we developed an siRNA strategy that after nucleofection reduced the levels of CXXC5 with 40–70% (see “Experimental Procedures”) but did not reduce Idax levels significantly in control cultures (Fig. 8A and data not shown). We then used this siRNA against CXXC5, named sir-CXXC5, in comparison with an unrelated siRNA against cyan fluorescent protein (sir-CFP) to investigate putative changes in the NSCs response to BMP4 and Wnt3a as assessed by RT-qPCR measurement of Axin2 gene expression levels after 4 h exposure. Interestingly, administration of sir-CXXC5 24 h before exposure of BMP4 resulted in an almost complete abolishment of the BMP4-mediated reduction of Axin2 levels (Fig. 8B). Cultures treated with sir-CXXC5 displayed around 10% reduction of Axin2 expression levels after BMP4 exposure, compared with an average of 60% reduction in cells that were nucleofected with sir-CFP (Fig. 8B). As the efficiency of the siRNA was significantly lower than 100% in our experiments (40–70%), a complete reversal of the BMP4/Wnt3a-mediated effects was not expected. Accordingly, sir-CXXC5 facilitated the increase in Axin2 levels in Wnt3a stimulated cultures compared with those cultures that received control siRNA (Fig. 8B). It should be noted that the Wnt3a-mediated increase in Axin2 was lower in all siRNA-transfected cultures than in any of the other experimental series, which we speculate is due to the siRNA treatment per se (40). We conclude that CXXC5 is a mediator of BMP4-mediated modulation of Wnt signaling in NSCs.

FIGURE 7. CXXC5 overexpression attenuates Wnt3a-mediated TOPflash reporter activity. A, relative luciferase activity of a TOPflash reporter construct in HEK-293 cells after co-transfections with empty (left bar) or β-catenin (right bar) expression vectors. Bars illustrate the average from two independent experiments in triplicates. B, relative luciferase activity of the TOPflash reporter construct in HEK-293 cells after treatment with 50 (left bars) or 100 (right bars) ng of purified Wnt3a and co-transfections with 0, 50 or 100 ng of CXXC5-containing expression vector. Note the marked decrease in reporter activity after co-transfection with 100 ng CXXC5 construct. Bars represent two independent experiments in triplicates. C, immunoblotting of total cell extracts from a fraction of the HEK-293 cells used in the corresponding luciferase experiments shown in B. No significant changes in total protein levels were seen after immunoblotting with β-catenin or Dvl antibodies. β-actin was used as loading control. D, immunoblotting of total cell extracts corresponding to C using an antibody against activated (dephosphorylated) β-catenin (ABC) compared with loading control (β-actin). The relative increase in ABC induced by Wnt3a is decreased in cells transfected with CXXC5.
CXXC5 Is a Neural BMP4-induced Modulator of Wnt Signaling

**DISCUSSION**

BMP4 treatment induces differentiation of NSCs in vitro into astrocytic and mesenchymal cells (4, 5) and is required for the proper development of the dorsal-most telencephalic structures, such as the hippocampal formation and the non-neuronal choroid plexus (1–3). In addition, BMP4 is in coordination with Wnt3a and FGF8 required for the correct size and regionalization of the dorsal pallium (1, 10). Yet little is understood regarding the cellular properties that allow and restrict these multiple and complex outcomes downstream of BMP4. In this study, we have identified CXXC5 as a novel potential target for cross-talk between BMP4 and canonical Wnt3a signaling as we show that CXXC5 is induced by BMP4 stimulation, selectively expressed in the BMP4-rich dorsal telencephalon, interacting with the Wnt signaling intermediate Dvl, and modulates the response to Wnt3a exposure in NSCs.

The telencephalic choroid plexus extends from the ventricular zone into the ventricles and produces and secretes the cerebrospinal fluid. Many non-neural cell types can be found in the choroid plexus, including epithelial and mesenchymal cells. Interestingly while Wnt3a expression is excluded from the developing choroid plexus, both BMP4 and CXXC5 expression levels are high in this region (Fig. 2 and Ref. 41). It appears that while Wnt3a is required for neuronal progression from these regions contributing to hippocampal structures, Wnt3a seems dispensable for choroid plexus development (1). A possible functional role for CXXC5 in forebrain development could therefore be to repress the effects of long range Wnt3a signaling to allow BMP4-dependent formation of the choroid plexus. Future studies using conditional gene deletion strategies specifically targeting CXXC5 in the developing dorsal telencephalon will be required to test this hypothesis.

The *Xenopus* homologue of the closely related factor Idax (CXXC4), named Xidax, has been shown to disrupt the correct formation of anterior brain structures when overexpressed in the developing *Xenopus* forebrain (13). This is not surprising considering its interaction with Dvl and thus interference with canonical Wnt signaling (11). The expression pattern of Idax in mammalian brain is not yet reported, but we have noted that Idax is expressed in telencephalic NSCs suggesting that it may be expressed also in the dorsal telencephalon. Although the sequence in the C-terminal parts of Idax and CXXC5 are very similar, the N-terminal domain shows clear divergences. Further, whereas Idax subcellular localization is preferentially cytoplasmic (11), CXXC5 is predominantly nuclear. It will therefore be of interest to investigate whether Idax and CXXC5 work synergistically, complementarily, or antagonistically. It has been demonstrated that the CXXC domain in the founding members of the CXXC family, the MBD proteins, participate in DNA and protein interactions (12). In addition, several of the other CXXC domain containing proteins have been shown to participate in chromatin modifying mechanisms and regulate and interfere with the activity of chromatin modifying factors, such as Polycomb proteins (12). It is therefore possible that some of the effects of CXXC5 are due to additional function/s in the nucleus, and ongoing studies are focusing on elucidating a potential role for nuclear CXXC5.

In summary, our results suggest that CXXC5 is a BMP4-regulated modulator of Wnt signaling essential for proper response of telencephalic stem cells to canonical Wnt3a signaling. We speculate that the functional role of CXXC5 in telencephalic development may be to contribute to the establishment of the demarcation between BMP4 and Wnt3a signaling regions during the development of hippocampal and choroid plexus formations.

Acknowledgments—We thank Per Uhlen for advice on FRET, Lars Björklund and the members of the Hermanson laboratory for discussions, John L. R. Rubenstein for constructs, and Christrer Höög, Claes Wahlestedt, Ole Isacson, and Jamie Timmons for support.

**REFERENCES**

1. Shimogori, T., Banuchi, V., Ng, H. Y., Strauss, J. B., and Grove, E. A. (2004) *Development* **131**, 5639–5647

2. Panchision, D. M., Pickel, J. M., Studer, L., Lee, S. H., Turner, P. A., Hazel, T. G., and McKay, R. D. (2001) *Genes Dev.* **15**, 2094–2110

3. Hebert, J. M., Mishina, Y., and McConnell, S. K. (2002) *Neuron* **35**, 1029–1041

4. Tsai, R. Y., and McKay, R. D. (2000) *J. Neurosci.* **20**, 3725–3735

5. Rajan, P., Panchision, D. M., Newell, L. F., and McKay, R. D. (2003) *J. Cell Biol.* **161**, 911–921

6. van Grunsven, L. A., Verstappen, G., Huylebroeck, D., and Verschueren, K. (2005) *Cytokine Growth Factor Rev.* **16**, 495–512

*T. Andersson and O. Hermanson, unpublished observations.*
CXXC5 Is a Neural BMP4-induced Modulator of Wnt Signaling

FEBRUARY 6, 2009 • VOLUME 284 • NUMBER 6
JOURNAL OF BIOLOGICAL CHEMISTRY
3681