Down-regulation of the Wnt/β-catenin signaling pathway by Cacnb4

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ABSTRACT The β_4 isoform of the β-subunits of voltage-gated calcium channel regulates cell proliferation and cell cycle progression. Herein we show that coexpression of the β_4-subunit with actors of the canonical Wnt/β-catenin signaling pathway in a hepatoma cell line inhibits Wnt-responsive gene transcription and decreases cell division, in agreement with the role of the Wnt pathway in cell proliferation. β_4-subunit–mediated inhibition of Wnt signaling is observed in the presence of LiCl, an inhibitor of glycogen synthase kinase (GSK3) that promotes β-catenin translocation to the nucleus. Expression of β_4-subunit mutants that lost the ability to translocate to the nucleus has no effect on Wnt signaling, suggesting that β_4-subunit inhibition of Wnt signaling occurs downstream from GSK3 and requires targeting of β_4-subunit to the nucleus. β_4-subunit coimmunoprecipitates with the TCF4 transcription factor and overexpression of TCF4 reverses the effect of β_4-subunit on the Wnt pathway. We thus propose that the interaction of nuclear β_4-subunit with TCF4 prevents β-catenin binding to TCF4 and leads to the inhibition of the Wnt-responsive gene transcription. Thereby, our results show that β_4-subunit is a TCF4 repressor and therefore appears as an interesting candidate for the regulation of this pathway in neurons where β_4-subunit is specifically expressed.

INTRODUCTION

The voltage-gated calcium channel (VGCC) is a heteromeric protein complex found not only at the plasma membrane of most excitable cells, but also in nonexcitable cells including lymphocytes (Badou et al., 2013). VGCCs are composed of a main pore-forming α_1-subunit generally associated with the auxiliary subunits β, $\alpha_2\delta$, and γ. Among these, the β-subunits exhibit an important set of functions such as the regulation of α_1-subunit trafficking to the plasma membrane (Bichet et al., 2000) and of its gating and kinetics properties (De Waard and Campbell, 1995). Ca^{2+} entering the cell through VGCC, in response to membrane depolarization, directly or indirectly participates in several organ-specific cellular events including muscle contraction, neurotransmitter release, cell division, and gene transcription (Deisseroth et al., 2003; Flavell and Greenberg, 2008;

Greer and Greenberg, 2008). As a consequence, mutations of any of the VGCC subunits that affect Ca^{2+} current are associated to various pathologies (Bidaud et al., 2006). However, during the past decade, accumulating evidence has revealed the direct involvement of the VGCC β-subunit (herein referred to as β-subunit) as well as of different domains of the $α_1$-subunit in processes that take place in cellular locations different from the plasma membrane. Although some of these functions may still be regulated by Ca^{2+}, they highlight the fact that both the β-subunit and $α_1$-subunit domains may act at a distance from the Ca^{2+} channel moiety and thus in a VGCC-independent manner.

Indeed, different isoforms of the β-subunit have been shown to control the transcription of different genes (Hibino et al., 2003; Zhang et al., 2010; Xu et al., 2011; Tadmouri et al., 2012, Ronjat et al., 2013). β-Subunits exert this effect by 1) interacting with and controlling the activity of various transcription factors including the thyroid hormone receptor TRα (Tadmouri et al., 2012) or Pax6c (Zhang et al., 2010) and 2) by recruiting proteins involved in DNA remodeling such as the heterochromatin protein 1 (Hibino et al., 2003; Xu et al., 2011; Tadmouri et al., 2012). The C-terminal domain of the pore-forming $α_1$-subunit has been shown to act as a transcription factor that controls expression of several genes (Gomez-Ospina et al., 2006; Du et al., 2013). In addition, some mutations of the $β_4$-subunit and of the C-terminal domain of the $α_{1c}$-subunit associated with human pathologies have been shown to affect their role in gene regulation, whereas they only mildly modify VGCC gating properties (Escayg et al., 2000; Watase et al., 2008). In a recent study, we showed that proliferation of CHO cells was inhibited by expression of the $β_4$-subunit. This effect required nuclear localization of the $β_4$-subunit because the expression of $β_4$-subunit mutants defective for the nuclear translocation has no effect on cell proliferation (Rima et al., 2017). We thus investigated whether the expression of $β_4$-subunit affects the Wnt/β-catenin signaling pathway, which is one of the fundamental mechanisms that control cell proliferation (for review, see Logan and Nusse, 2004, and Al-Harthi, 2012). The Wnt signaling pathway plays an important role during embryonic and brain development (Logan and Nusse, 2004; Mulligan and Cheyette, 2012), particularly in synapse formation and remodeling, dendritic growth and arborization, neurotransmission, neuroplasticity, neurogenesis, and neuroprotection (Maguschak and Ressler, 2012). In the absence of Wnt, β-catenin phosphorylation by the glycogen synthase kinase 3 (GSK3) promotes its degradation by the proteasome. Activation of the pathway induced by Wnt binding to the membrane receptor Frizzled (FZD) inhibits GSK3, resulting in the stabilization of β-catenin and its translocation to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) transcription factors. β-catenin thus relieves the action of TCF/LEF-associated repressors leading to the activation of Wnt-dependent genes transcription (MacDonald et al., 2009). TCF/LEF transcription factors bind to DNA consensus sequences referred to as the Wnt-responsive element (WRE) and control the transcription of many genes, among which are genes involved in cell proliferation such as Cyclin D1 (Graham et al., 2000; MacDonald et al., 2009; Cadigan and Waterman, 2012). Alteration of this pathway is associated to many pathologies including cancer and neurological diseases (MacDonald et al., 2009; Inestrosa et al., 2012), and a continuously increasing number of proteins are implicated in its regulation.

In this study, we extend our previous findings highlighting the role of the VGCC-$β_4$ subunit on cell proliferation and gene transcription by investigating its effect on the transcription of Wnt-responsive genes. Using a cell line harboring a constitutive level of Wnt/β-catenin activity, we show that heterologous expression of the $β_4$-subunit induces a strong inhibition of Wnt-responsive gene transcription. We show that this effect depends on the nuclear targeting of the $β_4$-subunit and on its interaction with the transcription factor of the TCF family. Our results show that the $β_4$-subunit acts as a repressor of TCF and competes with β-catenin for the binding to TCF.

RESULTS

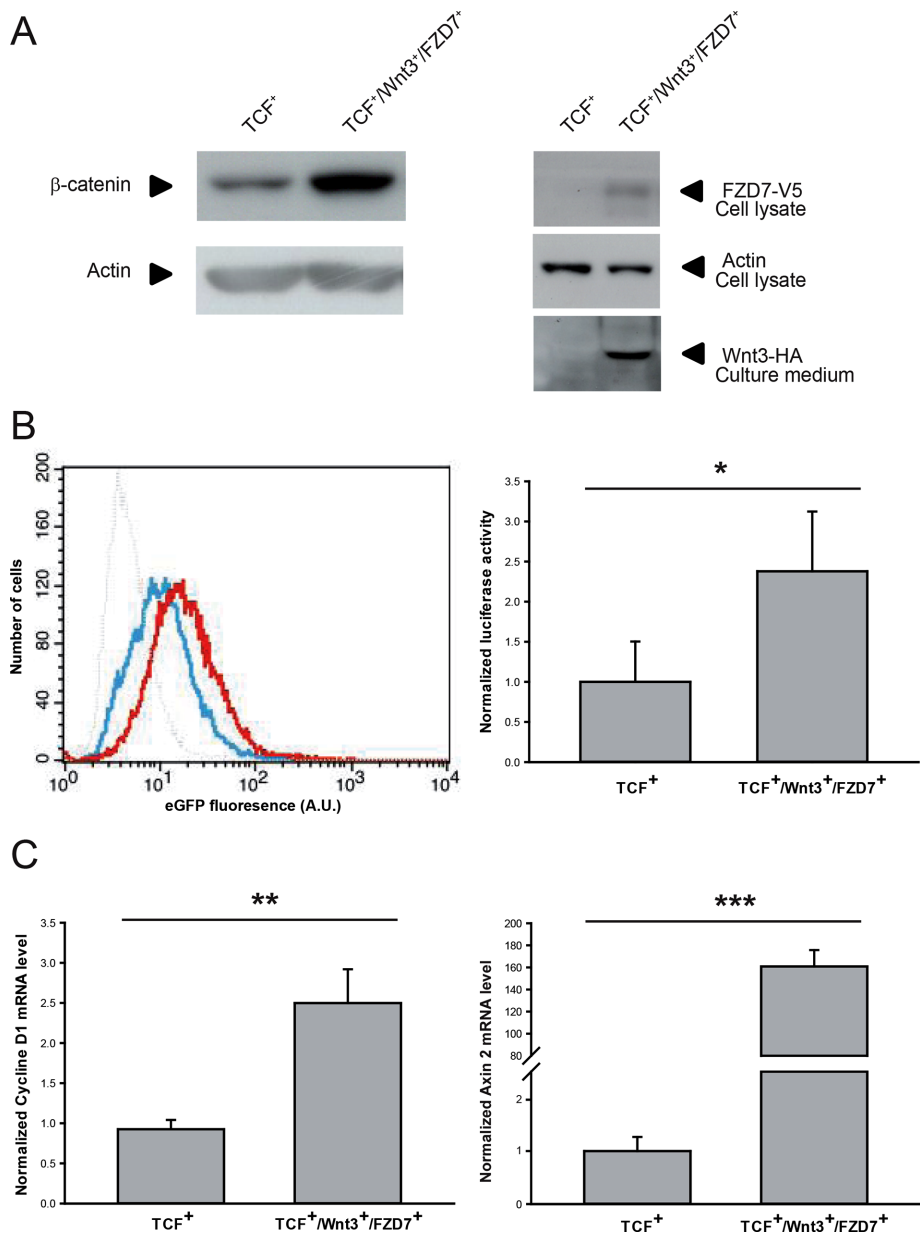To study the effect of the VGCC $β_4$-subunit on the Wnt/β-catenin pathway, we first generated a human hepatocellular carcinoma (HCC) FOCUS cell line, referred to as TCF[+], stably expressing a reporter gene system under the control of four WREs in tandem. FOCUS cells show a constitutive moderate activity of the Wnt/β-catenin pathway under the control of Wnt3 protein and Frizzled 7 plasma membrane receptor (FZD7) and do not present any mutation of the different elements of this pathway (Kim et al., 2008). In the absence of nuclear β-catenin accumulation, the TCF transcription factor binds to the WRE and represses the expression of the downstream reporter genes. When the Wnt signaling pathway is activated following the binding of Wnt to FZD, β-catenin translocates into the nucleus where it binds to TCF and relieves its transcription repressor activity leading to the activation of the downstream reporter genes. Two reporter genes were placed under the control of TCF encoding for luciferase and copGFP, respectively. A FOCUS cell line, referred to as TCF[−], expressing the same reporter gene system but missing the WRE sequences and thus insensitive to the Wnt/β-catenin pathway, was used as control. Finally, a cell line was generated that, in addition to the luciferase/copGFP reporter gene system, stably expresses both ectopic HA-tagged Wnt3 protein and V5 tagged FZD7. This cell line is referred to as TCF[+]/Wnt3^{+}/FZD7^{+}. The expression of both FZD-V5 and Wnt3-HA in TCF[+] and TCF[+]/Wnt3^{+}/FZD7^{+} cells is illustrated in Figure 1A (right panel). The activation of the Wnt/β-catenin pathway in the TCF[+]/Wnt3^{+}/FZD7^{+} cell line causes the increase of 1) β-catenin concentration (Figure 1A), 2) luciferase and copGFP fluorescence (Figure 1B), and 3) Cyclin D1 and Axin2 mRNA levels (Figure 1C).

$β_4$-Subunit inhibits cell proliferation

In a previous study, we showed that heterologous expression of the $β_4$-subunit in CHO cells inhibits cell proliferation (Rima et al., 2017). We thus investigated the effect of $β_4$-subunit expression on the proliferation of TCF[+]/Wnt3^{+}/FZD7^{+} cells using the Cell Proliferation Dye eFluor 670. To this end, cells were transfected with plasmids coding for $β_4$-eGFP or enhanced green fluorescent protein (eGFP) as a control. Twenty-four hours later, cells were labeled with the dye and the proliferation rate was assessed after 24, 48, and 72 h. The results show that the proliferation index of $β_4$-eGFP–expressing cells was significantly lower than those expressing eGFP (after 72 h, proliferation index = 16.2 ± 0.4 and 9 ± 0.2 for TCF[+]/Wnt3^{+}/FZD7^{+} cells expressing eGFP and $β_4$-eGFP, respectively, $p ≤ 0.0001$; Figure 2A). An assessment of $β_4$-subunit subcellular distribution in TCF[+]/Wnt3^{+}/FZD7^{+} cells shows that $β_4$-eGFP is mainly localized in the nucleus and accumulated within the nucleoli, whereas eGFP is evenly distributed in the nucleus and in the cytosol (Figure 2B).

$β_4$-Subunit regulates the transcriptional activity of the TCF promoter

We then investigated the effect of $β_4$-subunit expression on Wnt-responsive gene expression. To this end, the different cell lines

that do not overexpress Wnt3 and FZD7 (0.18 ± 0.06 A.U. for β_4-eGFP–expressing cells compared with 0.28 ± 0.03 A.U. for eGFP-expressing cells; $p \leq 0.0001$). In contrast, no significant change of luciferase activity was observed under expression of β_4-subunit in TCF$^-$ cells missing the WRE, indicating the specificity of the β_4-subunit inhibition of the Wnt pathway. A similar effect of β_4-subunit expression was observed by measuring copGFP transcription (Figure 3B). In this case, cells were transfected with β_4-myc encoding cDNA or empty plasmid as control and copGFP expression was quantified 24 h later. To test the specificity of the action of the β_4-subunit on the TCF promoter in HCC cells, we tested its effect on the serum-response factor (SRF)–dependent gene expression. HCC cells were transfected with a reporter gene system under the control of serum-response element (SRE) together with eGFP or β_4-eGFP. Sensitivity of this reporter gene system to the activation by SRF is highlighted by the increase of the SRE-luciferase activity when cells were incubated for 4 h in the presence of serum before measurement (Figure 3C). In contrast to what was observed in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells, expression of β_4-eGFP did not produce any significant change in SRE-luciferase activity either in the absence or in the presence of serum. To further confirm the inhibitory effect of β_4-subunit on the activity of the Wnt signaling pathway, we measured the endogenous expression of two well-known Wnt-responsive genes, *Cyclin D1* and *Axin2* (Shtutman et al., 1999; Jho et al., 2002), by quantitative real-time PCR (qRT–PCR). As shown in Figure 4A, the *Cyclin D1* mRNA level measured in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells significantly decreases in the presence of the β_4-subunit (1 ± 0.1 and 0.4 ± 0.1 in cells expressing eGFP and β_4-eGFP, respectively; $p \leq 0.01$), whereas transcription of *Axin2* is almost totally inhibited (1 ± 0.1 and 0.05 ± 0.1 in cells expressing eGFP and β_4-eGFP, respectively; $p \leq 0.0001$). Together, these observations suggest that the β_4-subunit partakes in the Wnt/β-catenin signaling pathway leading to the repression of the Wnt-responsive genes transcription.

FIGURE 1: Characterization of the TCF$^+$/Wnt3$^+$/FZD7$^+$ cell line. (A) Left panel: intracellular accumulation of β-catenin in TCF$^+$ and TCF$^+$/Wnt3$^+$/FZD7$^+$ cells. Right panel: expression of Wnt3-V5 and FZD7-HA in the TCF$^+$ and TCF$^+$/Wnt3$^+$/FZD7$^+$ cells. Cell lysate and culture medium were analyzed by SDS–PAGE and Western blot using anti–β-catenin, anti-V5 (Wnt3-V5), anti-HA (FZD7-HA), or anti-actin antibodies. (B) Left panel: increase of copGFP reporter gene expression in TCF$^+$/Wnt3$^+$/FZD7$^+$ was measured by flow cytometry. copGFP fluorescence signal measured in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells (red), TCF$^+$ cells (blue), and TCF$^-$ cells (gray; $n = 4$). Right panel: luciferase activity was measured in TCF$^+$ and TCF$^+$/Wnt3$^+$/FZD7$^+$ cells ($n = 4$). (C) *Cyclin D1* (left panel; $n = 3$) and *Axin2* (right panel; $n = 9$) mRNA expression level measured by RT-PCR in TCF$^+$ and TCF$^+$/Wnt3$^+$/FZD7$^+$ cell lines. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$.

described above were transfected with cDNA coding for β_4-eGFP or eGFP and luciferase expression was measured 24 h later (Figure 3A). β_4-subunit expression results in a twofold decrease of the luciferase expression in condition of ectopic boosting of the Wnt/β-catenin pathway, that is, in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells (0.44 ± 0.07 A.U. for β_4-eGFP–expressing cells compared with 0.95 ± 0.08 A.U. for eGFP-expressing cells; $p \leq 0.0001$). A significant decrease was also observed in cells expressing β_4-subunit and harboring basal steady-state Wnt/β-catenin pathway activation, that is, in TCF$^+$ cells

β_4-Subunit regulates the Wnt pathway downstream from GSK3

One of the key steps of the Wnt signaling pathway is the inhibition of GSK3 leading to the stabilization of β-catenin. To determine whether β_4-subunit regulation takes place upstream of or downstream from GSK3 inhibition, we measured the effect of the β_4-subunit under inhibition of GSK3 by LiCl. By inhibiting GSK3 activity, LiCl prevents β-catenin degradation and leads to its nuclear accumulation and the activation of Wnt-dependent genes, and therefore represents a

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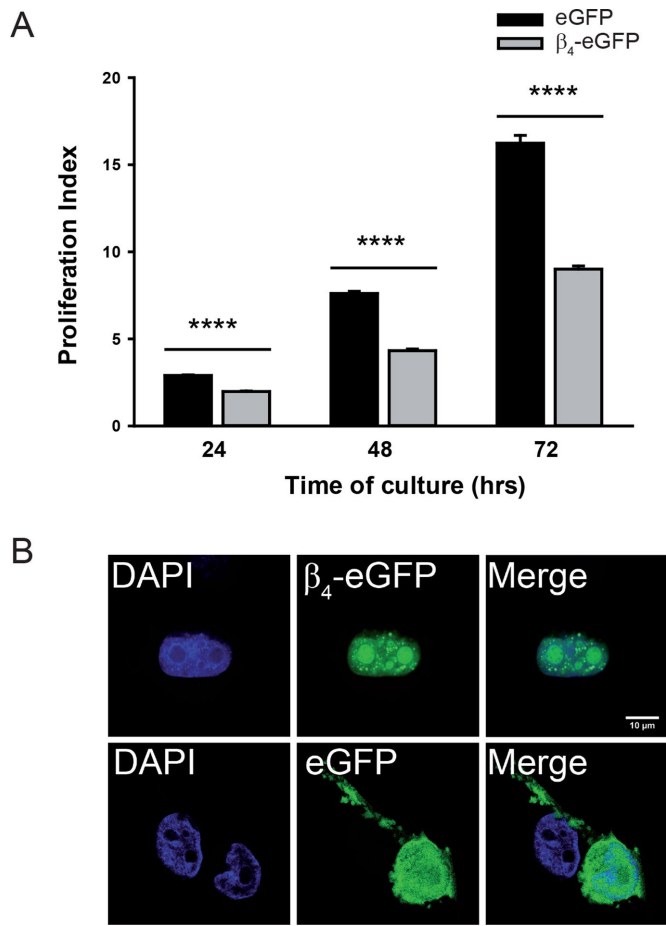
FIGURE 2: β_4-subunit expression inhibits the proliferation of TCF$^+$/Wnt3$^+$/FZD7$^+$ cells. (A) Proliferation index of TCF$^+$/Wnt3$^+$/FZD7$^+$ cells expressing eGFP (black bars) or β_4-eGFP (gray bars). The proliferation index was calculated using ModFit LT based on the decrease of Cell Proliferation Dye eFluor 670 signal ($n = 3$). ****, $p \leq 0.0001$. (B) Representative confocal images of TCF$^+$/Wnt3$^+$/FZD7$^+$ cells expressing β_4-eGFP or eGFP (green). Nuclei were visualized with DAPI (blue).

strong chemical activator of the Wnt signaling pathway (Klein and Melton, 1996). HCC TCF$^+$/Wnt3$^+$/FZD7$^+$ cells transfected with β_4-myc cDNA or with empty plasmid (pcDNA) were treated with LiCl (20 mM) for 24 h and copGFP expression was quantified at the end of the incubation. The results (Figure 4B) show that 1) LiCl induces an increase of copGFP expression as compared with NaCl treatment, showing the activation of the Wnt pathway and 2) the expression of the β_4-subunit significantly reduces the level of copGFP expression induced by LiCl. This finding suggests that the β_4-subunit inhibits the Wnt/β-catenin pathway downstream from GSK3, likely within the nucleus.

In a previous study, we showed that the structural integrity of the β_4-subunit is required for its translocation to the nucleus. Indeed, the β_4-subunit L125P mutation that is known to preclude the intramolecular interaction between the two globular SH3 and GK domains (McGee et al., 2004; Takahashi et al., 2005) completely abolishes its translocation to the nucleus (Tadmouri et al., 2012). Furthermore, the R482X nonsense mutation found in exon 14 introduces an upstream stop codon that causes the truncation of the last C-terminal 38 amino acids (Escayg et al., 2000). The loss of these 38 amino acids impairs β_4-subunit nuclear localization and its

ability to control gene transcription (Tadmouri et al., 2012; Ronjat et al., 2013). This variant (rs1805032) has been identified in a patient suffering a form of juvenile myoclonic epilepsy (Escayg et al., 2000) and is reported as a risk factor for the disease by ClinVar. Interestingly, although both mutations affect β_4-subunit nuclear localization, the L125P mutation abolishes its interaction with the α_1-subunit (McGee et al., 2004; Takahashi et al., 2004), whereas the R482X mutation only slightly affects the β_4-subunit interaction with the channel moiety (Escayg et al., 2000). We thus examined whether any of these two β_4-subunit mutations modify the effect of β_4-subunit expression on Wnt signaling. As shown in Figure 5A (top panel), β_{1-481}-eGFP mutant is defective for nuclear localization in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells (compared with β_4-eGFP; Figure 2B), whereas β_{L125P}-eGFP mutant is entirely excluded from the nucleus. Expression of β_{1-481}-eGFP or β_{L125P}-eGFP mutant in TCF$^+$/Wnt3$^+$/FZD7$^+$ and TCF$^-$/Wnt3$^+$/FZD7$^+$ cells did not affect the luciferase reporter gene activity (Figure 5B). These results show that the structural integrity of the β_4-subunit allowing its nuclear targeting is required for its effect on the expression of Wnt-responsive genes expression.

## β_4-Subunit interacts with the TCF4 transcription factor

As mentioned above, the main endpoint regulators of the Wnt signaling pathway are the TCF/LEF transcription factors. We thus investigated whether the β_4-subunit interacts with the TCF4 transcription factor. Using anti-GFP antibodies, we immunoprecipitated β_4-eGFP expressed in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells. Figure 6A (left panel) shows that endogenous TCF4 coprecipitates with β_4-eGFP demonstrating the presence of a β_4-eGFP/TCF4 complex. To test the β_4/TCF4 interaction in a tissue in which both proteins are endogenously expressed we immunoprecipitated endogenous β_4 from adult mice brain extract. Figure 6A (right panel) shows that endogenous TCF4 coimmunoprecipitates with endogenous β_4, indicating that β_4/TCF4 interaction indeed takes place in mice adult brain. Control immunoprecipitation using immunoglobulin G (IgG)-coated beads instead of anti-eGFP or anti–β_4-coated beads shows no precipitation of β_4-eGFP, β_4, or TCF4. Similarly, coimmunoprecipitation of the β_4-subunit and TCF4 was observed in CHO cells expressing both β_4-eGFP and TCF4-myc (Figure 6B, left panel). Immunoprecipitation with anti-eGFP antibodies in CHO cells expressing TCF4-myc and eGFP do not lead to precipitation of TCF4-myc (Figure 6B, right panel). In contrast to what is observed with β_4-eGFP, immunoprecipitation of β_{1-481}-eGFP with anti-GFP resulted in a very low level of TCF4-myc precipitation, whereas immunoprecipitation of β_{L125P}-eGFP did not lead to TCF4-myc precipitation. These results indicate that mutations of the β_4-subunit that perturb its nuclear localization also prevent its interaction with TCF4. Using a similar approach, we also investigated the interaction of the β_4-subunit with β-catenin as well as with Kaiso, a member of the BTB/POZ family of zinc finger transcription factors that has been shown to modulate the Wnt/β-catenin pathway (Park et al., 2005). No interaction of the β_4-subunit with any of these proteins was observed (unpublished data).

Overexpression of TCF4 counteracts β_4-subunit inhibitory effect of Wnt signaling

Because the β_4-subunit interacts with TCF4 but not with β-catenin, we hypothesized that the β_4-subunit and β-catenin compete for their interaction with TCF4. Direct, or indirect, interaction of the β_4-subunit with TCF4 would thus preclude β-catenin/TCF4 interaction and consequently the relief of TCF4 repression by TCF4 repressors, therefore maintaining the inhibition of Wnt-dependent genes
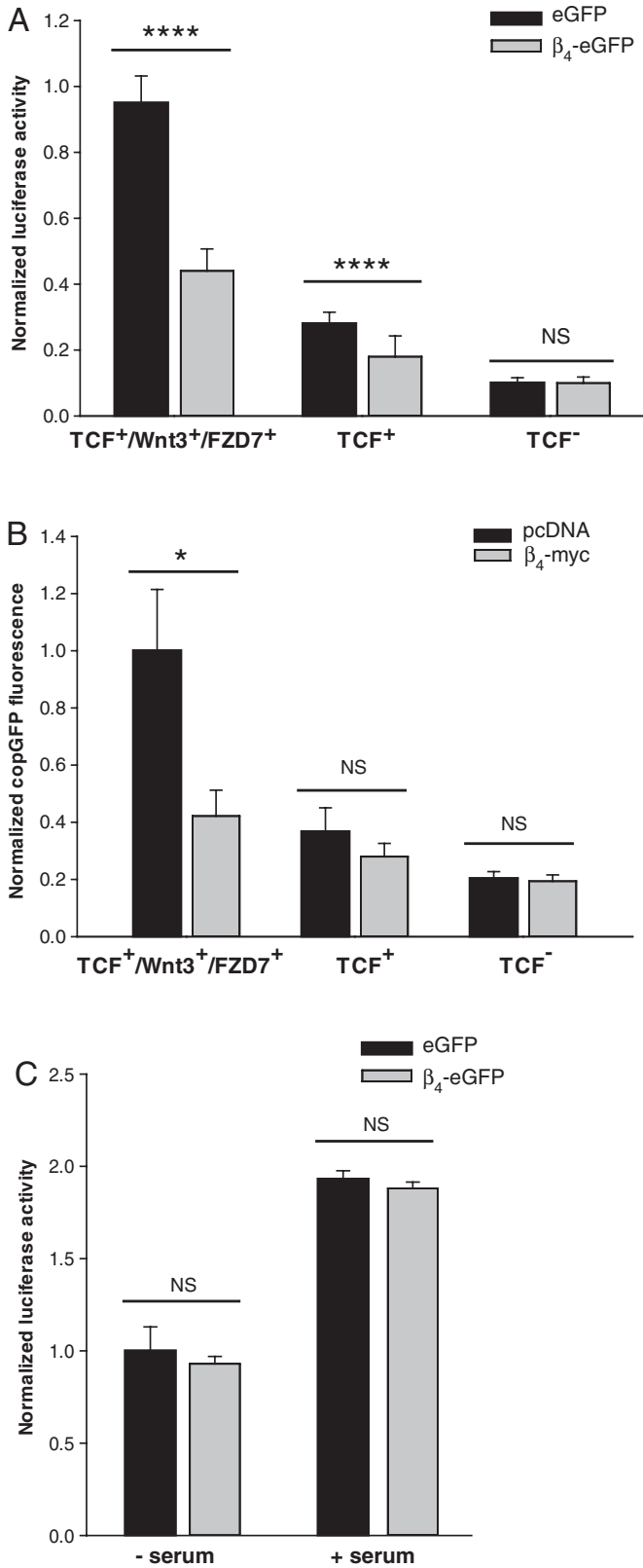
FIGURE 3: Effect of β_4-subunit on TCF and SRE-dependent transcription. (A) Luciferase activity measured in TCF$^+$/Wnt3$^+$/FZD7$^+$, TCF$^+$, and TCF$^-$ cells expressing eGFP (black bars) or β_4-eGFP (gray bars). (B) copGFP fluorescence measured in TCF$^+$/Wnt3$^+$/FZD7$^+$, TCF$^+$, and TCF$^-$ cells expressing pcDNA (black bars) or β_4-myc (gray bars). Luciferase and copGFP activity were normalized to the value measured in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells expressing eGFP (A) or pcDNA
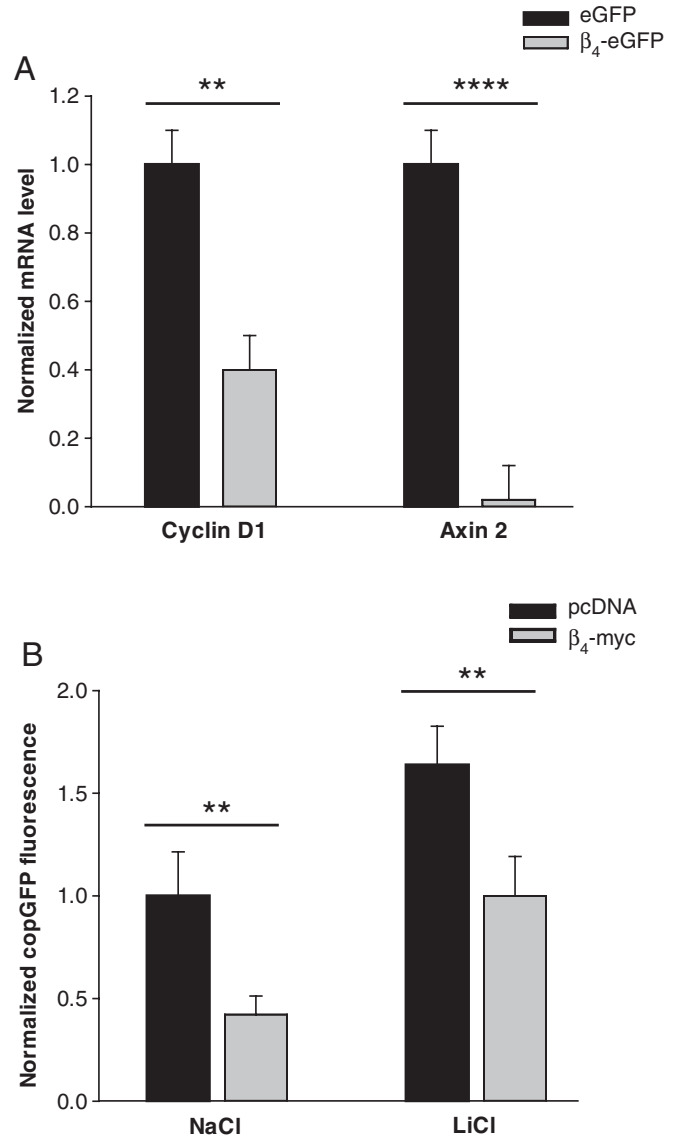

FIGURE 4: β_4-subunit inhibits the expression of Wnt-responsive genes. (A) *Cyclin D1* and *Axin2* mRNA expression level measured by qRT–PCR in TCF$^+$/Wnt3$^+$/FZD7$^+$ cells expressing eGFP (black bars) or β_4-eGFP (gray bars). Data were normalized using the *18s* as housekeeping gene. (B) copGFP expression measured after chemical activation of TCF$^+$/Wnt3$^+$/FZD7$^+$ cells with 20 mM LiCl. NaCl (20 mM) was used as control. Experiments were done in triplicate with $n = 3$. **, $p \leq 0.01$; ****, $p \leq 0.0001$.

(B). Experiments were done in triplicate with $n = 3$. (C) Luciferase activity measured in HCC cells expressing SRE reporter gene system, pTK-RL vector, and eGFP (black bars) or β_4-eGFP (gray bars). SRE-dependent luciferase activity was normalized to *Renilla* luciferase activity ($n = 2$). Transfected HCC cells were maintained in serum-free medium (− serum). Serum (10% final concentration) was added 4 h before luciferase measurement (+ serum). NS, nonsignificant; *, $p \leq 0.05$; ****, $p \leq 0.0001$.

VGCC β_4-subunit regulates Wnt signaling |
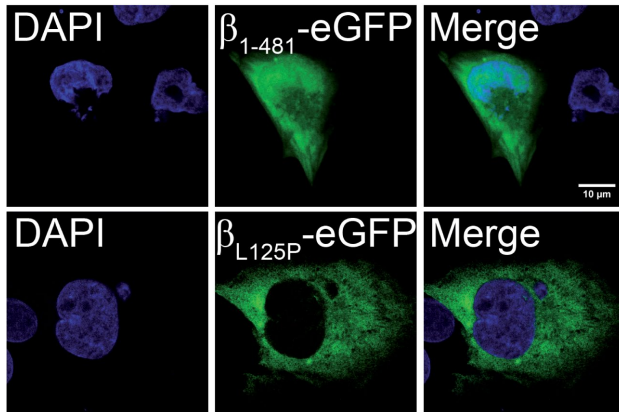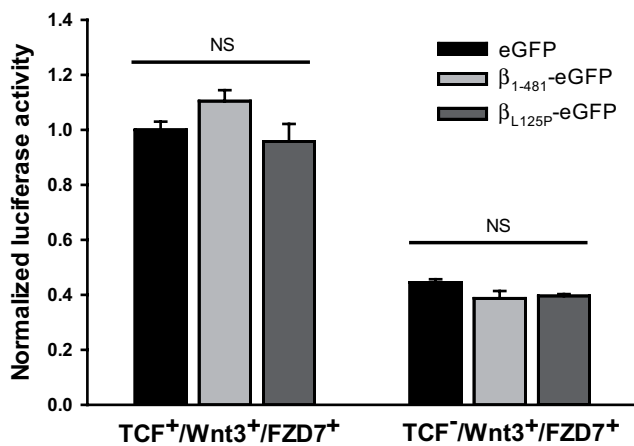
FIGURE 5: β₄-Subunit L125P and R482X mutations prevent its dependent inhibition of Wnt signaling. (A) Representative confocal images of TCF⁺/Wnt3⁺/FZD7⁺ cells expressing β_{1-481}-eGFP (green; top panel) or β_{L125P}-eGFP (green; bottom panel). Nuclei were labeled with DAPI (blue). (B) Luciferase expression in TCF⁺/Wnt3⁺/FZD7⁺ or TCF⁻/Wnt3⁺/FZD7⁺ cells expressing eGFP (black bars), β_{1-481}-eGFP (light-gray bars), or β_{L125P}-eGFP (dark-gray bars). Luciferase activity was normalized to its value in TCF⁺/Wnt3⁺/FZD7⁺ cells expressing eGFP ($n = 3$).

2017). Here, we describe the generation of a cell line stably expressing a double reporter gene (luciferase/copGFP) under the control of the WRE and TCF4. This cell line was further modified to stably express Wnt3 and FZD7, giving rise to a cell line exhibiting a constitutively activated canonical Wnt signaling pathway. Another cell line was generated that stably expresses the reporter genes but missing the WRE sequences, making the reporter genes insensitive to TCF4/β-catenin activation and thus to Wnt signaling. Using these cell lines we show that expression of β₄-subunit inhibits the expression of Wnt-responsive genes such as *Cyclin D1* and *Axin2*. *Cyclin D1* was previously identified as playing an important role in cell cycle progression (Baldin *et al.*, 1993). β₄-Subunit–induced inhibition of *Cyclin D1* expression could thus participate in the decrease of proliferation observed in cells expressing β₄-subunit. *Axin2* is a member of the Wnt-responsive gene family and its expression has been proposed to represent a negative feedback loop by favoring β-catenin degradation (Jho *et al.*, 2002).

In contrast to what we observed with wild-type β₄-subunit, expression of nuclear targeting deficient β₄-subunit mutants does not significantly modify β-catenin–mediated gene transcription. This result suggests that the effect of β₄ relies on both its structural integrity and nuclear localization. Finally, we demonstrate that β₄-subunit interacts with the transcription factor TCF4 and prevents the relief of its transcription repressor activity by β-catenin. The most established model for canonical Wnt signaling pathway relies on the fact that in the absence of Wnt, TCF4 binds to WRE sequences and acts as a transcriptional repressor for Wnt-responsive genes. This repressor activity is due to the interaction of TCF4 with a number of corepressor proteins that stabilize the transcriptional repression state of TCF4 (Ishitani *et al.*, 1999). Under activation of the Wnt pathway, β-catenin relocates to the nucleus and induces the unhooking of TCF4 corepressors allowing the functional remodeling of TCF4 that becomes a transcriptional activator. In this study, we show that β₄-subunit directly or indirectly interacts with TCF4 and that its inhibitory effect on Wnt-dependent genes transcription can be reversed by exogenous TCF4. We also show that the β₄-subunit/TCF4 complex also forms in the mice adult brain, reinforcing the physiological importance of this interaction. We thus propose that by interacting with TCF4, β₄-subunit prevents the recruitment of β-catenin and thus the removal of the corepressor proteins from TCF4. Whether the β₄-subunit/TCF4 interaction takes place within the cytoplasm or within the nucleus remains to be established. When TCF4 concentration is increased, a TCF4/β-catenin complex can be formed again leading to the activation of Wnt target genes. Our results show that β₄-subunit maintains TCF4 transcriptional repressor activity pointing to β₄-subunit as a member of the TCF4 corepressor family. The importance of the β₄-subunit/TCF4 interaction has to be considered in view of the high affinity of β-catenin for TCF4 (20 nM) that makes the identification of potent antagonists of this interaction difficult (Kahn, 2014).

We previously showed that β₄-subunit accumulates in the nucleoli. It has been recently proposed that the nuclear ratio between β-catenin and TCF is critical to ensure the optimal efficiency of Wnt signaling (Phillips and Kimble, 2009). It is thus also possible that, by sequestering TCF4 within the nucleoli, β₄-subunit decreases the concentration of nuclear TCF4 available for interacting with β-catenin and thus the concentration of β-catenin/TCF4 complexes. On the other hand, β₄-subunit interacts with B56δ, a regulatory subunit of PP2A phosphatase (Tadmouri *et al.*, 2012), and the phosphorylation/dephosphorylation process is known to control transcription factors activity (Wlodarchak and Xing, 2016). Indeed, the Traf2- and NCK-interacting kinase (TNIK) phosphorylates TCF3 and TCF4 (Shitashige *et al.*, 2010) leading to the activation of their

transcription. We thus tested whether β₄-subunit–induced inhibition of Wnt signaling can be reversed by overexpressing TCF4 in TCF⁺/Wnt3⁺/FZD7⁺ cells expressing β₄-subunit. As shown in Figure 7A, β₄-subunit–dependent inhibition of copGFP expression is prevented when TCF4-myc is coexpressed along with β₄-subunit. Figure 7B shows that β₄-subunit expression is not modified by the expression of TCF4. Therefore, excess of TCF4 is able to rescue the β-catenin–dependent transcriptional activity of TCF4, and consequently, to ride up copGFP expression to its level measured in the absence of β₄-subunit. These results further strengthen a model of competitive binding of β₄-subunit and β-catenin on TCF4.
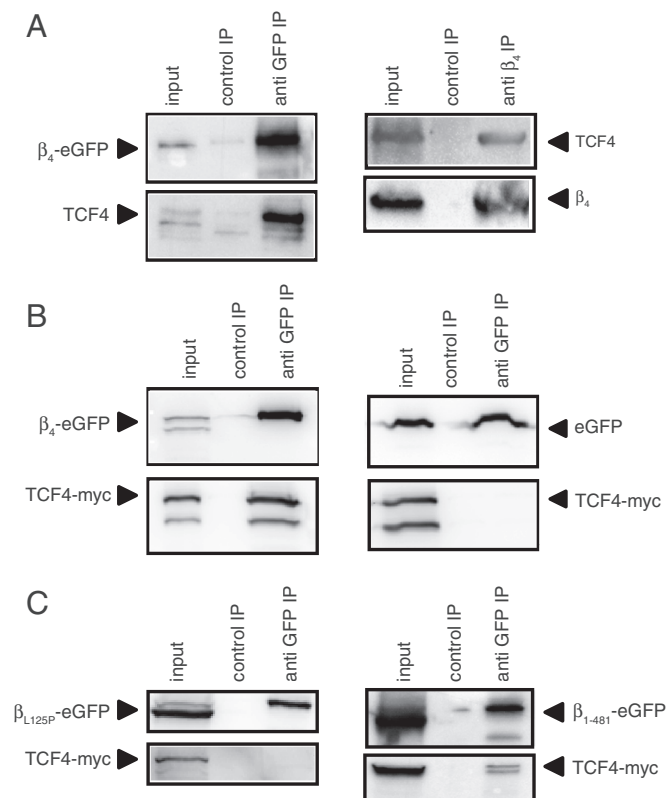
DISCUSSION

This study follows our previous findings establishing the role of the β₄-subunit in gene transcription regulation (Tadmouri *et al.*, 2012; Ronjat *et al.*, 2013) and its effect on cell proliferation (Rima *et al.*,

FIGURE 6: β4-Subunit interacts with the transcription factor TCF4. (A) Left panel: coimmunoprecipitation of β4-eGFP and endogenous TCF4 by anti-eGFP antibodies in TCF+/Wnt3+/FZD7+ cells. Right panel: antibodies directed against β4-subunit immunoprecipitate endogenous β4-subunit/TCF4 complex in adult brain mice extract. (B) Left panel: coimmunoprecipitation of β4-eGFP and TCF4-myc via anti-eGFP antibodies in CHO cells. Right panel: lack of coimmunoprecipitation shows the absence of TCF4-myc/eGFP interaction. (C) Left panel: immunoprecipitation of β_{L125P} does not induce precipitation of TCF4-myc. Right panel: β_{1-481}-eGFP immunoprecipitation poorly precipitates TCF4-myc. Control IP were done using IgG-coated beads.

transcriptional activity (Mahmoudi *et al.*, 2009). β4-Subunit, by targeting B56δ/PP2A to TCF4, could thus modify its phosphorylation state and as a consequence its effect on transcription.

β-Subunits have been described to interact with and control the activity of different transcription factors (Hibino *et al.*, 2003; Zhang *et al.*, 2010; Xu *et al.*, 2011; Tadmouri *et al.*, 2012). Our results add TCF4 to this list, suggesting that the control of transcription factor activity is an intrinsic property of β4-subunit. Wnt-responsive genes play a major role in basic cellular events such as cell proliferation, and abnormal Wnt signaling has been shown to be responsible for a number of pathologies including cancers (Polakis, 2012) and neurological diseases (Kalkman, 2012). Identification of proteins that control the action of β-catenin therefore represents an important milestone in the understanding of the tuning mechanisms of the Wnt signaling pathway in normal and pathological contexts.

MATERIALS AND METHODS
CHO cell culture
CHO cells were cultured in complete cell culture media containing DMEM/nutrient mixture F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (10 μg/ml).

Generation of focus cell lines stably expressing reporter genes under the control of TCF/LEF
The FOCUS (Ozturk *et al.*, 1987) HCC cell lines were maintained in DMEM supplemented with 10% fetal calf serum (FCS), 1% sodium pyruvate, 1% MEM nonessential amino acids, 1% glutamax, 100 μg/ml penicillin G, and 100 U/ml streptomycin. FOCUS cells have constitutive moderate activation of the Wnt/β-catenin under the control of the Wnt3 and FZD7 components, and absence of any mutations in the different elements of the pathway (CTNNB1, AXIN, APC, GSK3β; Kim *et al.*, 2008; Yuzugullu *et al.*, 2009). This cell line was manipulated in order to overexpress Wnt3 and FZD7 along with a TCF/LEF reporter gene. The reporter genes encoding for the Firefly luciferase and copGFP, respectively, are under the control of the murine cytomegalovirus (mCMV) minimal promoter, and four repetitions of the WRE sequence recognized by the transcription factors TCF/LEF are located upstream of the mCMV (TCF+ cells). A plasmid lacking the WRE sequences was used as negative control (TCF− cells). TCF+ and TCF− cells were transduced in order to stably express Wnt3 and FZD7 (TCF+/Wnt3+/FZD7+ cells and TCF−/Wnt3+/FZD7+, respectively).

### Plasmids and lentiviral transductions and TCF transcriptional activity assay
Human FZD7 cDNA was cloned into the plasmid plenti6/V5 directional TOPO with blasticidin resistance gene (Invitrogen; Nambotin *et al.*, 2011), resulting in a plenti6-FZD7/V5 plasmid encoding for a V5-tagged FZD7 membrane protein, and empty plenti6/V5 serving as negative control. The pUSEamp-WNT3 plasmid (Upstate) was subcloned into pLenti6/V5 directional TOPO plasmid, and thereafter into pBB/HA plasmid with hygromycin resistance gene, resulting in a pBB-WNT3/HA plasmid encoding for an HA-tagged Wnt3 excreted protein, and empty pBB/HA serving as negative control. All constructs were verified by sequence analysis of both strands. Virions were produced in human embryonic kidney (HEK) 293T cells (Invitrogen) and hepatoma cells were transduced at a multiplicity of infection of one (MOI-1). For the TCF transcriptional activity assay, plasmids PGF1-TCF/LEF-GFP (TR013PA-P) and PGF1-mCMV negative GFP control (TR010PA-P) with puromycin resistance gene (Gentaur) were used to produce virions. The corresponding lentiviruses were transduced at MOI-10. GFP-expressing cells were monitored by flow cytometry (FACSCalibur and CellQuestPro software). Luciferase-expressing cells were monitored with the luciferase assay systems E1500 (Promega).

Measure of β-catenin, FZD7, and Wnt3 expression
TCF+ and TCF+/Wnt3+/FZD7+ cells were harvested by scrapping, homogenized and sonicated in lysis buffer consisting of 30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% desoxycholate, 0.1% SDS, 2 mM EDTA, pH 7.5, 10% glycerol, 50 mM NaF, 2 mM Na3VO4, and 10 μl/ml complete protease inhibitor cocktail. Protein concentration was measured with the BCA reagent kit (Pierce). Western blotting was carried out using anti–β-catenin 1/500 (Santa Cruz), anti–β-actin 1/10,000 (Sigma) antibodies, and anti-V5 antibodies 1/5000 (Sigma) or anti-HA antibodies (Sigma). After adding the secondary horseradish peroxidase conjugated antibody, blots were visualized with the enhanced chemiluminescence detection system (Amersham).

Quantitative real-time RT-PCR (qRT-PCR)
Total RNA was extracted from cultured cells with Extract-all (Eurobio), whereas total RNA from FACS-sorted cells was extracted with
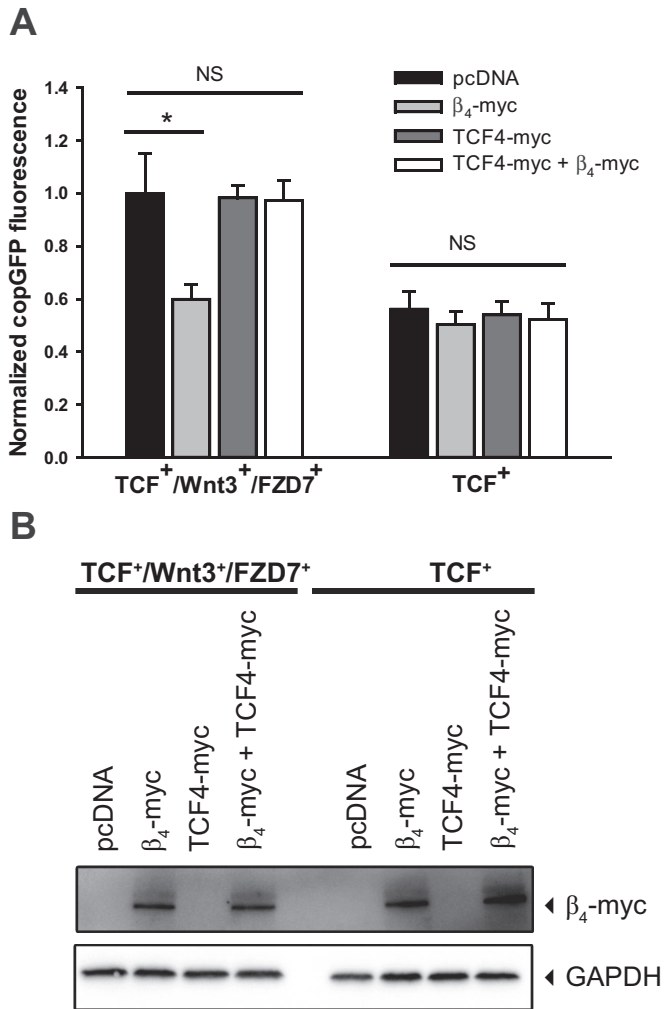
FIGURE 7: Heterologous expression of TCF4 rescues Wnt/β-catenin–dependent gene transcription in the presence of β4-subunit. (A) copGFP expression was measured by flow cytometry in TCF+/Wnt3+/FZD7+ and TCF+ cells expressing eGFP (black bars), β4-myc (light-gray bars), TCF4-myc (dark-gray bars), or TCF4-myc together with β4-myc (empty bars) ($n = 3$; *, $p \leq 0.05$). (B) Expression of β4-myc in the different conditions used in A. Identical volumes of cell extract from the different cells used in A were analyzed by SDS–PAGE and immunolabeling with antibodies directed against β4-subunit (top panel) or GAPDH (bottom panel).

Nucleospin RNA/XS (Macherey Nagel). M-MLV reverse transcriptase (Invitrogen) was used for cDNA synthesis after pretreatment by DNAse-I (Roche). PCRs were performed in the Light Cycler 480 (Roche), with a mix of 1X-Quantifast Qiagen SYBR Green, 500 nM each primer and 12.5 ng cDNA (equivalent total RNA). The thermal cycling conditions comprised an initial step of 5 min at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. Experiments were performed in triplicate. Each PCR run included standard curves and a nontemplate control. Analysis was performed using the comparative ΔCt method, and each gene normalized to the 18S RNA as housekeeping gene.

Twenty-four hours after transfection, cells, transfected with the plasmid coding for eGFP or β4-eGFP, were detached and sorted according to their eGFP fluorescence. eGFP-positive cells (200,000) were collected for each condition and their total RNA extracted as described above.

Sequences of the different primers designed using the Primer3 website are as follows: for *Axin2*, 5′-ATGGTGAGGTCCACGG-AAAC-3′ and 5′-CTTCACACTGCGATGCATTT-3′; for *Cyclin D1*, 5′-AAGGCGGAGGAGACCTGCGCG-3′ and 5′-ATCGTGCGGCA-TTGCGGC-3′ and for *18s* 5′-GGACACGGACAGGATTGACA-3′ and 5′-ACCCACGGAATCGAGAAAGA-3′.

Cell proliferation measurement

Cells were labeled with 2.5 µM Cell Proliferation Dye eFluor 670 (eBioscience) for 10 min at 37°C in the dark. The labeling was then stopped by adding five volumes of ice-cold complete media and incubating cells on ice for 5 min. At the end of the incubation period, cells were washed three times with complete media and analyzed by flow cytometry in a BD Accuri C6 system with a 640-nm laser. Various amounts of cells were then plated in 12WP to obtain 90% confluence after 24, 48, or 72 h of incubation. When the cultured cells reached 90% confluence, the cells were harvested, resuspended in DMEM/F-12 without phenol red, and the dilution of the fluorescent dye was assessed by flow cytometry. A minimum of 5000 events were analyzed for each sample. Obtained peaks were deconvoluted and the proliferation index was calculated using ModFit LT software.

Cell transfection

Expression vectors and cDNA constructs coding for pcDNA, eGFP, β4-eGFP (GenBank accession code L02315), β1-481-eGFP, βL125P-eGFP, B56δ-myc, and β4-myc were previously described (Tadmouri *et al.*, 2012). Plasmid coding for TCF4-myc and β-catenin were from Mireia Duñach (Universitat Autònoma de Barcelona, Departament de Bioquimica I de Biologia Molecular). The reporter construct SRE-Luc was a generous gift of Vincent Sauzeau (Inserm UMR 1087/CNRS, Nantes, France). CHO and FOCUS cells were transfected using Lipofectamine 2000 as per the manufacturer's instructions.

Luciferase and copGFP reporter assay

Twenty-four hours after transfection, cells were detached and sorted based on eGFP fluorescence as described above. An equal number of eGFP-positive cells for each condition was collected in 96-well plates. Firefly luciferase activity was then quantified with the Dual-Glo Luciferase Assay System according to manufacturer's instructions (Promega Corporation) using a PHERAstar FS microplate reader. SRE-Luc reporter luciferase activity was normalized to the *Renilla* luciferase activity of the cotransfected control vector pTK-RL (Promega).

When copGFP reporter gene expression was measured, cells were transfected with DNA coding for β4-myc, TCF4-myc, or pcDNA. Twenty-four hours after transfection, the cells were detached and analyzed by flow cytometry with a BD Accuri C6 system with a 488-nm laser. Relative fluorescence values were determined by analyzing a minimum of 5000 events for each sample. The data were analyzed using FCS express 5 and copGFP fluorescence intensity was expressed as the mode of the peaks.

Immunoprecipitation

Total proteins were extracted from cultured cells or total mouse brain using a lysis buffer consisting of 10 mM Tris (pH 7.5), 1.5 mM MgCl₂, 10 mM KCl, 0.5% Triton X-100, and protease inhibitor cocktail (Roche). Cells or brain were incubated in lysis buffer for 30 min on ice followed by sonication.

Before the incubation with the cell or brain extract, the magnetic Dynabeads Protein G (10003D, Thermo Fisher Scientific) were coupled with the immunoprecipitating antibody. Beads (50 µl) were washed three times with PBS-Tween 0.1%, resuspended in 50 µl of the washing buffer, and then incubated for 10 min with 10 µg of

anti-GFP antibody (11814460001, Roche) or anti β₄-subunit antibody (Everest Biotech) at room temperature under rotation. Antibody-coated beads were then washed twice with PBS-Tween 0.1%. Mouse IgG (M5284, Sigma)-coated beads were obtained using the same protocol and used as negative control. Protein extracts were added to antibodies coated beads and incubated for 30 min at room temperature under rotation. At the end of the incubation, the beads were rinsed three times with PBS-Tween 0.1% and finally resuspended in 15 µl of 5X Laemmli buffer for 10 min and then diluted with 60 µl of water. The samples were then heated for 10 min at 70°C and stored at −20°C.

Immunoprecipitated proteins were separated by SDS–PAGE in a 10% polyacrylamide resolving gel and then electrotransferred to Immobilon-P PVDF Membrane (Millipore). The membrane was blocked in 10% blotting-grade blocker (Bio-Rad) in PBS-Tween 0.1% for 1 h at room temperature and then incubated for 3 h with 1:5000 primary antibody: rabbit polyclonal anti-GFP (A-11122; Thermo Fisher Scientific), rabbit polyclonal anti-myc (ab9106; Abcam), rabbit polyclonal anti-TCF4 (ab185736; Abcam), or rabbit polyclonal anti-β₄ (Kiyonaka et al., 2007). After washing with PBS-Tween 0.1%, blots were incubated with HRP conjugated anti-rabbit IgG (sc-2004; Santa Cruz Biotechnology) or HRP conjugated protein A (ab7456; Abcam) at room temperature for 45 min. Immunolabeled proteins were visualized with the ECL detection method (Thermo Fisher Scientific).

Cell labeling

Cells cultured on glass coverslips were incubated with 5 µg/ml Hoechst 34580 (Thermo Fisher Scientific) for 5 min, washed with PBS, and fixed with 4% paraformaldehyde for 15 min. After being washed with PBS, cells were permeabilized with 0.1% Triton X-100. eGFP-tagged proteins were directly visualized by GFP fluorescence.

Samples were then mounted in ProLong Gold Antifade (P36931; Thermo Fisher Scientific) and images were acquired with a Nikon A1 Confocal microscope. Merged pictures were obtained by ImageJ-FiJi software.

Statistics

Student's two-tailed t test was used to calculate the statistical significance of differences between two sets of averaged data; $p \leq 0.05$ was considered statistically significant. Data are presented as mean ± SEM.

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