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

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ABSTRACT The β\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{4}-subunit mutants that lost the ability to translocate to the nucleus has no effect on Wnt signaling, suggesting that β\textsubscript{4}-subunit inhibition of Wnt signaling occurs downstream from GSK3 and requires targeting of β\textsubscript{4}-subunit to the nucleus. β\textsubscript{4}-subunit coimmunoprecipitates with the TCF4 transcription factor and overexpression of TCF4 reverses the effect of β\textsubscript{4}-subunit on the Wnt pathway. We thus propose that the interaction of nuclear β\textsubscript{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\textsubscript{4}-subunit is a TCF4 repressor and therefore appears as an interesting candidate for the regulation of this pathway in neurons where β\textsubscript{4}-subunit is specifically expressed.
As a consequence, mutations of any of the VGCC subunits that affect Ca\textsuperscript{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 \(\beta\)-subunit (herein referred to as \(\beta\)-subunit) as well as of different domains of the \(\alpha\)-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\textsuperscript{2+}, they highlight the fact that both the \(\beta\)-subunit and \(\alpha\)-subunit domains may act at a distance from the Ca\textsuperscript{2+} channel moiety and thus in a VGCC-independent manner.

Indeed, different isoforms of the \(\beta\)-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). \(\beta\)-Subunits exert this effect by 1) interacting with and controlling the activity of various transcription factors including the thyroid hormone receptor TR\(\alpha\) (Tadmouri et al., 2012) or Pax\(\alpha\)c (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 \(\alpha\)-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 \(\beta\)-subunit and of the C-terminal domain of the \(\alpha\)-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 \(\beta\)-subunit. This effect required nuclear localization of the \(\beta\)-subunit because the expression of \(\beta\)-subunit mutants defective for the nuclear translocation has no effect on cell proliferation (Rima et al., 2017). We thus investigated whether the expression of \(\beta\)-subunit affects the Wnt/\(\beta\)-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, neuromodulation, neurogenesis, and neuroprotection (Maguschak and Ressler, 2012). In the absence of Wnt, \(\beta\)-catenin phosphorylation by the glycosynthase 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 \(\beta\)-catenin and its translocation to the nucleus where it interacts with members of the T-cell factor (TCF)/lymphocyte enhancer factor (LEF) transcription factors. \(\beta\)-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-\(\beta\)\(_4\) 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/\(\beta\)-catenin activity, we show that heterologous expression of the \(\beta\)\(_4\)-subunit induces a strong inhibition of Wnt-responsive gene transcription. We show that this effect depends on the nuclear targeting of the \(\beta\)\(_4\)-subunit and on its interaction with the transcription factor of the TCF family. Our results show that the \(\beta\)\(_4\)-subunit acts as a repressor of TCF and competes with \(\beta\)-catenin for the binding to TCF.

**RESULTS**

To study the effect of the VGCC \(\beta\)\(_4\)-subunit on the Wnt/\(\beta\)-catenin pathway, we first generated a human hepatocellular carcinoma (HCC) FOCUS cell line, referred to as TCF\(_4\), stably expressing a reporter gene system under the control of four WREs in tandem. FOCUS cells show a constitutive moderate activity of the Wnt/\(\beta\)-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 \(\beta\)-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, \(\beta\)-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\(_4\), expressing the same reporter gene system but missing the WRE sequences and thus insensitive to the Wnt/\(\beta\)-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\(_4\)/Wnt3\(_3\)/FZD7\(_7\). The expression of both FZD-V5 and Wnt3-HA in TCF\(_4\) and TCF\(_4\)/Wnt3\(_3\)/FZD7\(_7\) cells is illustrated in Figure 1A (right panel). The activation of the Wnt/\(\beta\)-catenin pathway in the TCF\(_4\)/Wnt3\(_3\)/FZD7\(_7\) cell line causes the increase of 1) \(\beta\)-catenin concentration (Figure 1A), 2) luciferase and copGFP fluorescence (Figure 1B), and 3) Cyclin D1 and Axin2 mRNA levels (Figure 1C).

**\(\beta\)\(_4\)-Subunit inhibits cell proliferation**

In a previous study, we showed that heterologous expression of the \(\beta\)\(_4\)-subunit in CHO cells inhibits cell proliferation (Rima et al., 2017). We thus investigated the effect of \(\beta\)\(_4\)-subunit expression on the proliferation of TCF\(_4\)/Wnt3\(_3\)/FZD7\(_7\) cells using the Cell Proliferation Dye eFluor 670. To this end, cells were transfected with plasmids coding for \(\beta\)\(_4\)-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 \(\beta\)\(_4\)-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\(_4\)/Wnt3\(_3\)/FZD7\(_7\) cells expressing eGFP and \(\beta\)\(_4\)-eGFP, respectively, p ≤ 0.0001; Figure 2A). An assessment of \(\beta\)\(_4\)-subunit subcellular distribution in TCF\(_4\)/Wnt3\(_3\)/FZD7\(_7\) cells shows that \(\beta\)\(_4\)-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).

**\(\beta\)\(_4\)-Subunit regulates the transcriptional activity of the TCF promoter**

We then investigated the effect of \(\beta\)\(_4\)-subunit expression on Wnt-responsive gene expression. To this end, the different cell lines

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that do not overexpress Wnt3 and FZD7 (0.18 ± 0.06 A.U. for β4-eGFP-expressing cells compared with 0.28 ± 0.03 A.U. for eGFP-expressing cells; p ≤ 0.0001). In contrast, no significant change of luciferase activity was observed under expression of β4-subunit in TCFβ4 cells missing the WRE, indicating the specificity of the β4-subunit inhibition of the Wnt pathway. A similar effect of β4-subunit expression was observed by measuring copGFP transcription (Figure 3B). In this case, cells were transfected with β4-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 β4-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 β4-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 TCFβ4/Wnt3+/FZD7* cells, expression of β4-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 β4-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 TCFβ4/Wnt3+/FZD7* cells significantly decreases in the presence of the β4-subunit (1 ± 0.1 and 0.4 ± 0.1 in cells expressing eGFP and β4-eGFP, respectively; p ≤ 0.01), whereas transcription of Axin2 is almost totally inhibited (1 ± 0.1 and 0.05 ± 0.1 in cells expressing eGFP and β4-eGFP, respectively; p ≤ 0.0001). Together, these observations suggest that the β4-subunit partakes in the Wnt/β-catenin signaling pathway leading to the repression of the Wnt-responsive genes transcription.

β4-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 β4-subunit regulation takes place upstream of or downstream from GSK3 inhibition, we measured the effect of the β4-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
strong chemical activator of the Wnt signaling pathway (Klein and Melton, 1996). HCC TCF/\(\beta\)-catenin/LEF1 cells transfected with \(\beta\)-catenin/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 \(\beta\)-subunit significantly reduces the level of copGFP expression induced by LiCl. This finding suggests that the \(\beta\)-subunit inhibits the Wnt/\(\beta\)-catenin pathway downstream from GSK3, likely within the nucleus.

In a previous study, we showed that the structural integrity of the \(\beta\)-subunit is required for its translocation to the nucleus. Indeed, the \(\beta\)-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 \(\beta\)-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 \(\beta\)-subunit nuclear localization, the L125P mutation abolishes its interaction with the \(\alpha\)-subunit (McGee et al., 2004; Takahashi et al., 2004), whereas the R482X mutation only slightly affects the \(\beta\)-subunit interaction with the channel moiety (Escayg et al., 2000). We thus examined whether any of these two \(\beta\)-subunit mutations modify the effect of \(\beta\)-subunit expression on Wnt signaling. As shown in Figure 5A (top panel), \(\beta\)_L125P-eGFP mutant is defective for nuclear localization in TCF/\(\beta\)-catenin cells (compared with \(\beta\)_eGFP; Figure 2B), whereas \(\beta\)_R482X-eGFP mutant is entirely excluded from the nucleus. Expression of \(\beta\)_R482X-eGFP or \(\beta\)_L125P-eGFP mutant in TCF/\(\beta\)-catenin and TCF/\(\beta\)-catenin cells did not affect the luciferase reporter gene activity (Figure 5B). These results show that the structural integrity of the \(\beta\)-subunit allowing its nuclear targeting is required for its effect on the expression of Wnt-responsive genes expression.

**\(\beta\)_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 \(\beta\)-subunit interacts with the TCF4 transcription factor. Using anti-GFP antibodies, we immunoprecipitated \(\beta\)_4-eGFP expressed in TCF/\(\beta\)-catenin cells and found that \(\beta\)_4-eGFP interacts with the TCF4 transcription factor (Figure 6A, left panel). Immunoprecipitation with anti-GFP antibodies in CHO cells expressing both \(\beta\)_4-eGFP and TCF4-myc (Figure 6B, left panel) shows that endogenous TCF4 coprecipitates with \(\beta\)_4-eGFP indicating that \(\beta\)_4/TCF4 interaction indeed takes place in mice adult brain. Control immunoprecipitation using immunoglobulin G (IgG)-coated beads instead of anti-eGFP or anti-\(\beta\)_4-coated beads shows no precipitation of \(\beta\)_4-eGFP, \(\beta\)_4, or TCF4. Similarly, communoprecipitation of the \(\beta\)-subunit and TCF4 was observed in CHO cells expressing both \(\beta\)_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 \(\beta\)_4-eGFP, immunoprecipitation of \(\beta\)_L125P-eGFP gave precipitates with TCF4-myc resulting in a very low level of TCF4-myc precipitation, whereas immunoprecipitation of \(\beta\)_R482X-eGFP did not lead to TCF4-myc precipitation. These results indicate that mutations of the \(\beta\)-subunit that perturb its nuclear localization also prevent its interaction with TCF4. Using a similar approach, we also investigated the interaction of the \(\beta\)-subunit with \(\beta\)-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/\(\beta\)-catenin pathway (Park et al., 2005). No interaction of the \(\beta\)-subunit with any of these proteins was observed (unpublished data).

**Overexpression of TCF4 counteracts \(\beta\)-subunit inhibitory effect of Wnt signaling**

Because the \(\beta\)-subunit interacts with TCF4 but not with \(\beta\)-catenin, we hypothesized that the \(\beta\)-subunit and \(\beta\)-catenin compete for their interaction with TCF4. Direct, or indirect, interaction of the \(\beta\)-subunit with TCF4 would thus preclude \(\beta\)-catenin/TCF4 interaction and consequently the relief of TCF4 repression by TCF4 repressors, therefore maintaining the inhibition of Wnt-dependent genes
β4-subunit regulates Wnt signaling

FIGURE 3: Effect of β4-subunit on TCF and SRE-dependent transcription. (A) Luciferase activity measured in TCF+/Wnt3+/FZD7+, TCF+, and TCF− cells expressing eGFP (black bars) or β4-eGFP (gray bars). Data were normalized using the 18s as housekeeping gene. (B) copGFP fluorescence measured in TCF+/Wnt3+/FZD7+, TCF+, and TCF− cells expressing pcDNA (black bars) or β4-myc (gray bars). Luciferase and copGFP activity were normalized to the value measured in TCF+/Wnt3+/FZD7+ cells expressing eGFP (A) or pcDNA (B). Experiments were done in triplicate with n = 3. **, p ≤ 0.01; ****, p ≤ 0.0001.

FIGURE 4: β4-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 β4-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 ≤ 0.01; ****, p ≤ 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 β4-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 ≤ 0.05; ****, p ≤ 0.0001.
transcription. We thus tested whether β4-subunit-induced inhibition of Wnt signaling can be reversed by overexpressing TCF4 in TCF4+/Wnt3+/FZD7+ cells expressing β4-subunit. As shown in Figure 7A, β4-subunit–dependent inhibition of copGFP expression is prevented when TCF4-4myc 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., 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 subunit 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 mouse 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., 2010), 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
transcriptional activity (Mahmoudi et al., 2009). β4-Subunit, by targeting B56β/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 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; Yuzugul 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+ and TCF−/Wnt3+/FZD7+, respectively).

Plasmids and lentiviral transductions and TCF transcriptional activity assay

Human FZD7 cDNA was cloned into the plasmid plent6/V5 directional TOPO with blasticidin resistance gene (Invitrogen; Nambotin et al., 2011), resulting in a plent6-FZD7/V5 plasmid encoding for a V5-tagged FZD7 membrane protein, and empty plent6/V5 serving as negative control. The pUSEamp-WNT3 plasmid (Upstate) was sub-cloned into pLent6/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 (Gentauro) 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 scraping, homogenized and sonicated in lysis buffer consisting of 30 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% NP40, 0.5% deoxycholate, 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, biots 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
were collected for each condition and their total RNA extracted as
according to their eGFP fluorescence. eGFP-positive cells (200,000)

A

- compares the expression levels of Wnt3, FZD7, and TCF4 in the different conditions used in A. Identical volumes of cell extract

B

- immunolabeling with antibodies directed against

Nucleospin RNA/XS (Macherey Nagel). M-MLV reverse transcriptase
(Invitrogen) was used for cDNA synthesis after pretreatment by DN-
Ase-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,

- MgCl2 brain using a lysis buffer consisting of 10 mM Tris (pH 7.5), 1.5 mM

- Immunoprecipitation

- Total proteins were extracted from cultured cells or total mouse

- Ten minutes after transfection, the cells were detached and sorted

- Twenty-four hours after transfection, the cells were detached and sorted

- Luciferase and copGFP reporter assay

- Expression vectors and cDNA constructs coding for pcDNA, eGFP,

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- Expression vectors and cDNA constructs coding for pcDNA, eGFP,

- Twenty-four hours after transfection, cells were detached and sorted

- Sequences of the different primers designed using the Primer3

- 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 in-
cubating 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.

- Twenty-four hours after transfection, cells were detached and sorted

- Cell transfection

- Expression vectors and cDNA constructs coding for pcDNA, eGFP,

- Luciferase and copGFP reporter assay

- Twenty-four hours after transfection, cells were detached and sorted

- Immunoprecipitation

- Total proteins were extracted from cultured cells or total mouse

- Twenty-four hours after transfection, the cells were detached and sorted

- Expression vectors and cDNA constructs coding for pcDNA, eGFP,

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- Expression vectors and cDNA constructs coding for pcDNA, eGFP,

- Luciferase and copGFP reporter assay

- Twenty-four hours after transfection, cells were detached and sorted

- Immunoprecipitation

- Total proteins were extracted from cultured cells or total mouse

- Twenty-four hours after transfection, the cells were detached and sorted

- Expression vectors and cDNA constructs coding for pcDNA, eGFP,
anti-GFP antibody (11814460001, Roche) or anti β3-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 re-suspended 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 electrophoresed to Immobilon-P PVDF Membrane (Millipore). The membrane was blocked in 10% blocking-buffer (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; Thermofisher Scientific), rabbit polyclonal anti-myc (ab9106; Abcam), rabbit polyclonal anti-TCF4 (ab185736; Abcam), or rabbit polyclonal anti-β3 (Kiyonaka et al., 2007). After washing with PBS-Tween 0.1%, blots were incubated with HRP conjugated anti-rabbit IgG (sc-2004; Santa Cruz Biotechology) or HRP conjugated protein A (ab74566; 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 ≤ 0.05 was considered statistically significant. Data are presented as mean ± SEM.

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