The cellular prion protein PrPc is a partner of the Wnt pathway in intestinal epithelial cells

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Running head: Intestinal nuclear PrPc and Wnt pathway

Abbreviations: DKK1: Dickkopf-related protein 1; GPI: glycosylphosphatidyl inositol; PrPc: cellular prion protein; PLA: proximity ligation assay; TCF/LEF: T cell factor/lymphoid enhancer factor; TCF7L2: transcription factor 7-like 2; YAP: Yes-associated protein.

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Abstract

We reported previously that the cellular prion protein (PrP<sup>c</sup>) is a component of desmosomes and contributes to the intestinal barrier function. We demonstrated also the presence of PrP<sup>c</sup> in the nucleus of proliferating intestinal epithelial cells. We aimed to decipher the function of this nuclear pool. In human intestinal cancer cells Caco-2/TC7 and SW480 and normal crypt-like HIEC-6 cells, PrP<sup>c</sup> interacts, in cytoplasm and nucleus, with γ-catenin, one of its desmosomal partner, and with β-catenin and TCF7L2, the main effectors of canonical Wnt pathway. PrP<sup>c</sup> up-regulates the transcriptional activity of the β-catenin/TCF7L2 complex while γ-catenin down-regulates it. Silencing of PrP<sup>c</sup> results in the modulation of several Wnt target gene expression in human cells, with different effects depending on their Wnt signaling status, and in mouse intestinal crypt cells in vivo. PrP<sup>c</sup> interacts also with the Hippo pathway effector YAP, suggesting that it may contribute to the regulation of gene transcription beyond the β-catenin/TCF7L2 complex. Finally, we demonstrate that PrP<sup>c</sup> is required for proper formation of intestinal organoids, indicating that it contributes to proliferation and survival of intestinal progenitors. In conclusion, PrP<sup>c</sup> must be considered as a new modulator of the Wnt signaling pathway in proliferating intestinal epithelial cells.
Introduction

The cellular prion protein (PrP<sup>c</sup>) has been studied essentially for its ability to undergo a structural conversion into a pathogenic conformer, which is a key process for the onset of transmissible spongiform encephalopathies (Prusiner, 1998). Beside this role in prion diseases, PrP<sup>c</sup> is expressed in a wide range of tissues and cell types, where it has been shown to contribute to the regulation of many basic biological processes, including cell proliferation, differentiation, survival and adhesion (Westergard et al., 2007). It participates to several specific functions in specialized tissues, such as neuroprotection, synaptic activity, olfaction, immune response, epithelial and endothelial barrier function (Linden et al., 2008; Petit et al., 2013). PrP<sup>c</sup> was described to be secreted mostly as a glycosylphosphatidyl inositol (GPI)-anchored glycoprotein associated to lipid raft microdomains of the plasma membrane (Vey et al., 1996; Naslavsky et al., 1997), where it combines with several protein partners, including signaling molecules (Mouillet-Richard et al., 2000; Morel et al., 2004; Santuccione et al., 2005). The ability of PrP<sup>c</sup> to modulate cell signaling was proposed to mediate at least some of its biological effects (for reviews: (Westergard et al., 2007; Linden et al., 2008)). However, the complete repertoire of PrP<sup>c</sup> biological functions has not been determined and the analyses of the different subcellular localizations of the protein as well as the identification of its site-specific partners are important to achieve this goal.

Among the various extra-neuronal tissues that express PrP<sup>c</sup>, previous studies from our group focused on intestine, the first site for infectious agent entry into the organism. In enterocytes, which represent the most abundant population of intestinal epithelial cells, we demonstrated that PrP<sup>c</sup> is addressed to cell-cell junctions (Morel et al., 2004), where it interacts with several desmosomal proteins (desmoglein-2, desmoplakin, γ-catenin (plakoglobin) and plakophilin-2) and participates to the structure of desmosomes (Morel et al., 2008). We showed that PrP<sup>c</sup> is required for the proper organization of adherens and tight junctions as well, thereby contributing to the intestinal barrier function (Petit et al., 2012). We reported also a shortening of intestinal villi in PrP knock out mice (Morel et al., 2008), thus suggesting an involvement of PrP<sup>c</sup> in intestinal epithelium homeostasis.

The intestinal epithelium undergoes a rapid renewal throughout life (Stappenbeck et al., 1998), which requires a continuous coordination between proliferation, differentiation and death programs. Stem cells and dividing transit amplifying cells migrate from the crypt to the villus where most of differentiated cells are located. In proliferating intestinal epithelial cells, i.e. in the intestinal crypts in vivo or in dividing human Caco-2/TC7 enterocytes in culture, we demonstrated that a PrP<sup>c</sup> pool was unexpectedly localized in the nucleus (Morel et al., 2008). A nuclear localization of the prion protein was reported also in neuronal cells for both the protease-resistant PrP form in prion infected cells (Mange et al., 2004) and the normal PrP<sup>c</sup> in non-infected cells (Hosokawa et al., 2008). A nuclear localization signal (NLS) was
described in the N-terminal domain of the mature PrP. However, this sequence was not efficient in targeting Green Fluorescent Protein toward the nuclear compartment (Jaegly et al., 1998), and the neurotoxic truncated 23-230 PrP was shown addressed to the nucleus independently of its nuclear localization signals (Crozet et al., 2006). Although the mechanisms leading to the nuclear targeting of normal or pathological prion proteins remain unknown, PrPc was found associated with the lectin CBP70 in the nucleus of NB4 human promyelocytic leukemia cell line (Rybner et al., 2002) or with chromatin (Mange et al., 2004), histone H3, H1-0 and lamin B1 in neuronal and β cells of the endocrine pancreas (Strom et al., 2011). These later findings suggested a role of PrPc in transcriptional regulation, but PrPc function in the nucleus had to be deciphered.

To attain this objective, we first characterized the nuclear partners of PrPc through a proteomic approach and identified γ-catenin, a component of desmosomes in differentiated cells, as a nuclear PrPc partner in proliferating intestinal epithelial cells. γ-catenin is a protein of the catenin family displaying a dual junctional and nuclear localization (Aktary and Pasdar, 2012). γ-catenin is homologous to β-catenin, the core player of the canonical Wnt pathway, which drives cell proliferation in intestinal crypts and is involved in intestinal homeostasis (Pinto et al., 2003; Clevers and Nusse, 2012). β-catenin is localized at adherens junctions where it interacts with E-cadherin cytodomain. In the absence of Wnt stimulation, cytoplasmic β-catenin is phosphorylated by a destruction complex, which involves casein kinase 1 (CK1), glycogen synthase kinase 3β (GSK3β), adenomatous polyposis Coli (APC) and axin, and addressed to the proteasome for degradation. In response to Wnt activation, the destruction complex is disrupted, leading to the stabilization of the cytoplasmic β-catenin and its targeting to the nucleus where it acts as a co-activator of the transcription factors of the TCF/LEF family to regulate the expression of many target genes involved in a variety of cellular processes (Clevers and Nusse, 2012). Although γ-catenin is known to interact with the TCF/LEF transcription factors, its exact role in the Wnt pathway is much less documented than that of β-catenin and seems to vary between different biological systems (Aktary and Pasdar, 2012).

The identification of PrPc as a new γ-catenin nuclear partner led us to investigate whether it could interfere with the canonical Wnt pathway. Therefore, we analyzed if PrPc interacts with the Wnt signaling effectors β-catenin and TCF7L2 (also known previously as TCF4), the major member of the TCF family in intestine, and studied how PrPc impacts on β-catenin/TCF7L2 transcriptional activity, on the expression of Wnt target genes or on cell proliferation.

Results
The nuclear PrPc interacts with γ-catenin in proliferating Caco-2/TC7 enterocytes

As a first approach to determine the role of PrPc when it is addressed to the nucleus, a proteomic analysis was realized after immunoprecipitation of PrPc from nuclear extracts of exponentially growing Caco-2/TC7 cells, in which we demonstrated previously the nuclear localization of a PrPc pool (Morel et al., 2008). This analysis revealed that the partners of PrPc in the nucleus included desmoplakin and γ-catenin (plakoglobin), identified previously as PrPc desmosomal partners in differentiated enterocytes (Petit et al., 2012), along with desmoglein 1 preproprotein, Glyceraldehyde-3-Phosphate Dehydrogenase, Squamous cell carcinoma antigen (SCCA2/SCCA1) and γ-actin (Table 1).

We further focused on the interaction between PrPc and γ-catenin. The nuclear co-localization of PrPc and γ-catenin was confirmed by confocal microscopy in exponentially growing Caco-2/TC7 cell clusters (Figure 1A). Both proteins were detected in the nucleus (arrowheads) and in the cytoplasm. It must be noticed that a part of γ-catenin was also localized at cell-cell contacts, as was PrPc (arrows) but with a much lower intensity, suggesting that PrPc needs a better maturation of cell-cell junctions than γ-catenin to be targeted to the membrane.

PrPc and γ-catenin were detected in nuclear extracts by western blot analysis (Figure 1B) and PrPc/γ-catenin interaction was attested by co-immunoprecipitation (Figure 1C). We further confirmed that these two proteins interact in the nucleus using in situ proximity ligation assay (PLA) and confocal microscopy (Figure 1D). This approach showed that PrPc/γ-catenin interaction occurred mainly in the nucleus of proliferating Caco-2/TC7 cells. By contrast, this interaction was detected predominantly at cell-cell contacts in differentiated cells (Figure 1D), as observed also for the interaction between γ-catenin and another desmosomal protein, desmoplakin, as expected (not shown). The precision and reliability of this assay to localize interactions of endogenous proteins were assessed by the absence of PLA signal at cell-cell contacts between PrPc and the adherens junction-associated E-cadherin, which colocalize by immunofluorescence but do not interact, as we showed previously (Morel et al., 2004); in the same way, no PLA signal was detected in the nucleus between PrPc and the transcription factor HNF-4 α (Supplementary Figure 1).

Characterization of the expression and subcellular localization of PrPc, γ-catenin and the Wnt effectors β-catenin and TCF7L2 (TCF4) in different intestinal cell lines

The identification of PrPc as a new γ-catenin nuclear partner led us to investigate the possible links between this complex and the canonical Wnt pathway. We therefore analyzed the expression of PrPc and γ-catenin, as well as that of β-catenin and TCF7L2, the major end point effectors of the canonical Wnt signaling in intestine, during the exponential growth
phase of several intestinal cell lines. With respect to their basal Wnt pathway activities, we selected three intestinal cell-lines for this study. They comprised the two cancer cell-lines SW480, with a constitutively high activity of Wnt pathway and Caco-2/TC7, in which DKK1 overexpression attenuates the constitutive Wnt signaling (Aguilera et al., 2007), and the nontumoral crypt-like human intestinal cells HIEC-6, with a weak basal activity of Wnt pathway (Guezguez et al., 2014). Consistent with the Wnt pathway status, β-catenin protein level was much higher in SW480 cells than in the two other cell lines (Figure 2A). No correlation was observed between Wnt activity and PrPγ protein levels, which were similar in HIEC-6 and SW480 cells and higher than in Caco-2/TC7 cells. Interestingly, the highest γ-catenin level was detected in Caco-2/TC7 cells, which display the lowest β-catenin and PrPγ levels (Figure 2A). TCF7L2 level was very low in HIEC-6 cells and two-fold higher in Caco-2/TC7 than in SW480 cells. PrPγ, γ-catenin and β-catenin were present in the nuclear fraction of the three cell lines (Figure 2B). Confocal microscopy analyses confirmed the nuclear localization of the four proteins, but highlighted differences in their subcellular partitioning between the cell lines. PrPγ, γ-catenin and β-catenin were visualized in the nucleus and the cytoplasm in both SW480 and HIEC-6 cells, but with a much higher nuclear proportion in SW480 than in HIEC-6 cells (Figure 3). By contrast, even at this very low-density stage, Caco-2/TC7 cells exhibited a partial junctional localization of PrPγ, γ-catenin and β-catenin, in accordance with the well-described ability of these cells to polarize, to establish mature cell-cell junctions and to differentiate (Chantret et al., 1994), contrary to the two other cell lines. The transcription factor TCF7L2 was concentrated mostly in the nucleus in SW480 and Caco-2/TC7 cells, as expected, but was much more diffusely distributed between the cytoplasm and the nucleus in HIEC-6 cells.

PrPγ and γ-catenin interact with the Wnt effectors β-catenin and TCF7L2 in proliferating intestinal cells

γ-catenin interacts with the transcription factor TCF7L2 (Miravet et al., 2002). We hypothesized that PrPγ could thus participate to molecular complexes involving γ-catenin, β-catenin and TCF7L2. The PLA approach was chosen in order to visualize protein interactions and their subcellular distribution in situ. In exponentially growing SW480, Caco-2/TC7 and HIEC-6 cells, PrPγ interacts with γ-catenin, β-catenin and TCF7L2 (Figure 4). γ-catenin/TCF7L2 and β-catenin/TCF7L2 interactions were detected also, as expected, and the presence of γ-catenin/β-catenin interactions confirmed the existence of multi-partner complexes reported previously (Miravet et al., 2002). In SW480 and Caco-2/TC7 cells, the PLA fluorescent spots were concentrated in the nucleus for all interactions, although they could be observed also in the cytoplasm. By contrast, but in agreement with the distribution
of all partners that was observed by immunofluorescence (Figure 3), interactions between PrP\textsuperscript{c}/\gamma-catenin, PrP\textsuperscript{c}/\beta-catenin and \gamma-catenin/\beta-catenin were in much higher proportions in the cytoplasm than in the nucleus of HIEC-6 cells (Figure 4). In this latter cell line, complexes involving TCF7L2 were very rare, in accordance with its low level (Figures 2 and 3).

**PrP\textsuperscript{c} interaction with TCF7L2 depends mostly on its \beta-catenin binding domain**

To determine whether PrP\textsuperscript{c} interacts with TCF7L2 via \beta- or \gamma-catenin, or independently of these proteins, we transfected SW480 cells with different fragments of a FLAG-TCF7L2 protein (Figure 5). Fragment expression was analyzed by immunofluorescence and protein interactions were studied by PLA. Interactions of \beta-catenin, \gamma-catenin and PrP\textsuperscript{c} with the full length FLAG-TCF7L2 were easily detected in transfected cells, although the PLA signal intensity reported to FLAG expression was much higher for \beta-catenin/FLAG-TCF7L2 interaction than for \gamma-catenin/ or PrP\textsuperscript{c}/FLAG-TCF7L2 interactions (Figure 5A). These results were in accordance with the differences observed for interactions of each protein with the endogenous TCF7L2 (Figure 4). When FLAG-TCF7L2 constructs were deleted for the binding domains of \beta-catenin (del 1-51) or of both \beta- and \gamma-catenin (del 2-100), their interaction with \beta-catenin was completely lost (Figure 5B). This result is in agreement with the GST-pull down approach that was used previously to identify these binding sites (Miravet et al., 2002), thus demonstrating the accuracy of the PLA technique. The deletion of the catenin binding domains decreased interactions of TCF7L2 with PrP\textsuperscript{c} by 85% and 90% for del 1-51 and del 2-100 constructs respectively and with \gamma-catenin by 80% for both constructs. Interactions with \beta-catenin were restored partially for the construct deleted for the 52-143 fragment, and totally for the construct deleted for the 82-143 fragment, as expected. Very similar results were obtained for interactions of PrP\textsuperscript{c} with these constructs. By contrast, interactions with \gamma-catenin remained very low with the del 52-143 construct, as expected, and were only partially restored with the del 82-143 fragment, probably because this latter deletion impacts on the conformation of \gamma-catenin binding domain.

Interactions between \beta-catenin, or PrP\textsuperscript{c}, and TCF7L2 can occur in the cytoplasm, as shown by analyses of TCF7L2 mutants obtained by serial deletions of approximately 100 amino acids from the C-terminal part of the protein, comprising or not the NLS (Supplementary Figure 2). Interactions of TCF7L2 with both proteins were detected mostly in the nucleus with fragments 1-500 and 1-420, in the cytoplasm and around the nucleus with the 1-307 fragment, which is devoid of NLS, but were concentrated again in the nucleus with the 1-201 fragment, which is small enough to diffuse inside this compartment (Supplementary Figure 2).
The above results showed that PrP\(_c\) interaction with TCF7L2 depends mostly on the \(\beta\)-catenin binding domain. To further determine whether PrP\(_c\) interacts with TCF7L2 through \(\beta\)-catenin, we studied the impact of \(\beta\)- or \(\gamma\)-catenin silencing by small interfering RNA (siRNA) on PrP\(_c\)/TCF7L2 interactions in both nuclear and cytoplasmic compartments. Upon silencing of \(\beta\)-catenin, which was decreased by more than 90% in both cytoplasm and nucleus (Figure 6A), PrP\(_c\)/TCF7L2 interactions were significantly decreased (Figure 6B). This loss of interactions concerned the nucleus compartment (-25%), with no impact on cytoplasmic interactions (Figure 6B, compare hatched bars with white bars). However, this decrease of nuclear interactions was modest, most of them being maintained in the absence of \(\beta\)-catenin. \(\gamma\)-catenin silencing (-80% for total level, -70% for nuclear level, Figure 6A) had no impact on PrP\(_c\)/TCF7L2 interactions either in the cytoplasm or the nucleus (Figure 6B). Moreover, the invalidation of one catenin had no significant effect on the interaction of PrP\(_c\) with the other catenin.

**PrP\(_c\) up-regulates the transcriptional activity of the \(\beta\)-catenin/TCF7L2 complex**

We then addressed the functional role of PrP\(_c\) in the Wnt signaling pathway using a transcriptional reporter assay and compared it to that of \(\gamma\)-catenin, which has been suggested to exert a negative effect on TCF7L2-mediated transcription (Miravet 2002). TOP or FOP reporter plasmids were co-transfected in COS7 cells together with a constitutively activated form of \(\beta\)-catenin and either PrP\(_c\) or \(\gamma\)-catenin. PrP\(_c\) increased the luciferase activity induced by \(\beta\)-catenin in a dose-dependent manner, whereas \(\gamma\)-catenin decreased it (Figure 7A). In SW480 cells, which have a constitutive high \(\beta\)-catenin level, we confirmed these opposite effects of PrP\(_c\) and \(\gamma\)-catenin (Figure 7B). The positive regulation of the transcriptional activity of the \(\beta\)-catenin/TCF7L2 complex by PrP\(_c\) was further demonstrated by the decreased luciferase activity upon PrP\(_c\) silencing by siRNA and its rescue upon co-transfection with mouse PrP\(_c\), which is not impacted by the siRNA (Figure 7C; for siRNA efficiency, see Figure 8A and supplementary Figure 3).

**PrP\(_c\) knockdown affects the expression of Wnt and Hippo target genes**

PCR array analyses were then performed to evaluate changes in the expression of a large panel of Wnt target genes in response to PrP\(_c\) or \(\gamma\)-catenin silencing. Experiments were conducted on SW480 and HIEC-6 cells, which differ greatly in their Wnt pathway activity levels. Caco-2/TC7 cells were not analyzed because the presence of a desmosome-associated pool of PrP\(_c\) and \(\gamma\)-catenin in these cells, even at very early stages of the culture, could render the results difficult to interpret. Efficiency of silencing by siRNA was attested by a net decrease of the corresponding mRNA levels (Figure 8A). Protein levels were
diminished by 70 to 90% in both total cell lysates and nuclear extracts of SW480 or HIEC-6 cells from 24 hours after transfection (Supplementary Figure 3). This processing time was then chosen for further analyses.

Among the 84 Wnt target genes that were analyzed, the expression of 29 was modulated in at least one cell-line upon either PrP$c$ or $\gamma$-catenin siRNA treatment (Figure 8B). Surprisingly, PrP$c$ silencing in SW480 cells resulted in the increased expression of 15 genes (≥1.4 fold), with no decreased gene expression (≤0.7 fold) (Figure 8C). The expression of half of these 15 genes was increased also upon PrP$c$ silencing in HIEC-6 cells, in which basal Wnt activity is very low, but, in addition, the expression of 7 genes was lowered in these cells (Figure 8C). The impact of $\gamma$-catenin silencing was similar to that of PrP$c$ silencing for most of the genes whose expression was modulated in HIEC-6 cells, but differed markedly from that of PrP$c$ silencing in SW480 cells (Figure 8B). The classical transcriptional TCF7L2 targets, MYC, CCND1 and AXIN2, were not significantly modulated by PrP$c$ or $\gamma$-catenin silencing, even though the expression of the three genes tended to increase when analyzed by qPCR in SW480 cells (Supplementary Figure 4).

To determine whether PrP$c$ could interfere with the expression of Wnt target genes in vivo, the mRNA level of 8 genes, whose expression was modulated in cell-lines (Figure 8B), as well as that of Myc and Ccnd1, was analyzed in wild type or PrP knockout mice, in the bottom of intestinal crypts where high Wnt activity is observed in vivo (Figure 8D). The expression of 7 genes was increased in crypt cells in the absence of PrP$c$. They include Myc and Ccnd1, whose expression was not significantly modulated in cell lines, Sox9, Ets2, Irs1 and Gja1, whose expression was increased in SW480 cells upon PrP$c$ silencing, but also Id2, whose expression was decreased in HIEC-6 only. The expression of Igf2, Fgf9 and Tcf4 (new nomenclature) was not modified. None of the tested genes showed a decreased expression in crypt cells of PrP knockout as compared to wild type mice.

The apparently conflicting data in TOP/FOP reporter assays and PCR array analyses, as well as the impact of PrP$c$ or $\gamma$-catenin silencing on the expression of Wnt target genes in HIEC-6 cells, in which nuclear $\beta$-catenin and TCF7L2 levels are very low, indicate that PrP$c$ or $\gamma$-catenin could modulate Wnt target gene expression through other transcriptional effectors. As some of these genes are also transcriptional targets of the YAP/TEAD complex, an effector of the Hippo pathway (Zhao et al., 2008), we analyzed whether interaction between PrP$c$ and YAP could occur. Confirming this hypothesis, PLA analyses revealed the presence of PrP$c$/YAP complexes both in the cytoplasm and nucleus of proliferating HIEC-6, Caco-2/TC7 and SW480 cells (Figure 9).
**PrP c is required for proper intestinal organoid formation**

The Wnt pathway is a driving force for cell proliferation in intestinal crypts (Clevers and Nusse 2012). The up-regulation of the β-catenin/TCF7L2 transcriptional activity by PrP c prompted us to examine a possible effect of PrP c on cell proliferation. We observed previously a transient arrest of cell proliferation after PrP c knockdown by siRNA in Caco-2/TC7 cells (Morel et al., 2008). We confirmed this result in HIEC-6 cells, but not in SW480 cells, in which, however, PrP c overexpression induced a slight increase in cell proliferation (data not shown). We thought that the possible role of PrP c in cell proliferation would be studied more accurately in a model of normal intestinal progenitor cells. We isolated crypts from wild type and PrP knockout mice and performed intestinal organoid cultures (Sato et al., 2009). Figure 10A shows a net decrease of frequency in organoid formation from PrP knockout as compared to wild type mice. The few organoids that were obtained displayed the expected organization, with a regular E-cadherin and β-catenin membrane staining (Figure 10C) and accumulation of apoptotic cells in the internal lumen (not shown), but were in general smaller (Figure 10B), and showed less developed crypt domains (Figure 10B and C) than the organoids developed from the wild type crypts.

These results could be linked to a weaker Wnt activity in intestinal epithelial crypt cells from PrP knockout mice. We compared β-catenin nuclear staining in the bottom of intestinal crypts from wild type and PrP knockout mice, and observed a decreased frequency of cells exhibiting a clear nuclear localization of β-catenin in PrP KO crypts (Figure 11).

**Discussion**

We identified the nuclear partners of PrP c in proliferating intestinal epithelial cells and unraveled a new role for this protein in the modulation of Wnt pathway. PrP c interacts not only with γ-catenin, one of its desmosomal partner (Morel et al., 2008), but also with β-catenin and TCF7L2, which are the main effectors of Wnt pathway in intestinal cells, the β-catenin binding domain of TCF7L2 being crucial for its interaction with PrP c. Furthermore, we demonstrate that PrP c modulates the expression of several Wnt target genes, has a positive impact on the transcriptional activity of the β-catenin/TCF7L2 complex and is required for establishing intestinal organoids ex vivo.

We showed the presence of a nuclear pool of PrP c in two adenocarcinoma cell lines, SW480 and Caco-2/TC7 cells, as well as in normal crypt-like cells HIEC-6 (Figure 2), in accordance with our previous results in human intestinal crypts (Morel et al., 2008). However, it must be noticed that PrP c was less concentrated in the nucleus of HIEC-6 cells, which exhibit a very low Wnt activity, than in the two other cell-lines, in which the effectors of Wnt pathway are present in the nucleus (Figure 3). Since PrP c lacks a functional NLS (Jaegly
et al., 1998), it likely needs a partner to be imported into the nucleus. TCF7L2 could be such a partner, as it was suggested for β-catenin (Shitashige et al., 2008). By the use of PLA, which allows visualizing the subcellular localization of protein interactions, we show that PrPc can interact with β-catenin, γ-catenin and TCF7L2 outside the nucleus. This is observed for the interactions of endogenous proteins (Figure 4) and for PrPc or β-catenin interactions with TCF7L2 constructs lacking their NLS (supplementary Figure 2). PrPc interactions with β-catenin, γ-catenin and TCF7L2 were concentrated in nucleus only in the context of high Wnt activity and TCF7L2 levels, i.e. in SW480 and Caco-2/TC7 cells and not in HIEC-6 cells (Figure 4). These results suggest that multi-partner complexes are formed in the cytoplasm before their nuclear import via the classical NLS of TCF7L2.

Combining the use of TCF7L2 mutants and β-catenin silencing (Figures 5 and 6), we show that β-catenin mediates part of PrPc/TCF7L2 interactions through the β-catenin binding domain of TCF7L2, but that other intermediate proteins and/or a direct interaction between PrPc and TCF7L2 may also exist. By contrast, the presence of the γ-catenin binding domain of TCF7L2 has no influence on its interaction with PrPc and, accordingly, γ-catenin silencing does not modulate these interactions (Figures 5 and 6), even though PrPc/γ-catenin and γ-catenin/TCF7L2 complexes are observed (Figures 1 and 4). Finally, our results in HIEC-6 cells suggest that PrPc and β-catenin may interact also independently of TCF7L2 (Figure 4). Involvement of PrPc in these multiple combinations of protein complexes, whose composition most probably differs between cytoplasm and nucleus, could modify either the nuclear import of the Wnt effectors or their activity in the nucleus.

The transcription factor complex β-catenin/TCF7L2 is responsible for the transcriptional modulation of Wnt target gene expression in intestinal epithelial cells (Hatzis et al., 2008). The γ-catenin/TCF7L2 complex was shown to be inefficient in binding to DNA (Zhurinsky et al., 2000; Miravet et al., 2002). It was suggested previously that γ-catenin exerts by itself a negative regulation of TCF7L2 transcriptional activity (Miravet et al., 2002), but, through its ability to displace β-catenin from adherens junctions or from the destruction complex, can also enhance the β-catenin/TCF7L2 pool (Salomon et al., 1997; Aktary and Pasdar, 2012). In the present study, we observed a negative modulation of TCF7L2 transcriptional activity by γ-catenin. By contrast, we establish for the first time a positive regulation of this activity by PrPc (Figure 7). Since activation of the Wnt pathway is necessary for proliferation of intestinal epithelial cells, this result is in agreement with 1) the impairment of growth and survival of intestinal organoids from PrP knockout mice (Figure 10) and 2) the alteration of nuclear β-catenin localization in intestinal crypts of PrP knockout mice (Figure 11). This could explain the shortening of the villi that we described previously in PrPc knockout mice (Morel et al., 2008) and is in accordance with the positive role of PrPc on
gastric cancer cell proliferation mediated by PI3K/Akt (Liang et al., 2007), a pathway that may interfere with several steps of Wnt signalling (Yan and Lackner, 2012).

In this context, the increased expression of several Wnt target genes upon PrPc silencing seems contradictory, in particular, in crypts of PrP knockout mice, the up-regulation of Myc and Ccnd1, which are well known to mediate effects of Wnt signaling on cell proliferation (van de Wetering et al., 2002). Compensatory effects in knockout mice cannot explain these discrepancies since the same tendency was observed in SW480 cells shortly after PrPc invalidation. In HIEC-6 cells, in which nuclear β-catenin and TCF7L2 levels are very low, PrPc and γ-catenin are able to modulate the expression of genes identified as Wnt targets, suggesting that both proteins interfere with the activity of other transcriptional effectors as well. Multiple crosstalks between the Wnt and Hippo pathways have been unraveled recently (Azzolin et al., 2012; Rosenbluh et al., 2012). In this study, we showed an interaction of PrPc with the transcriptional co-activator Yes-associated protein (YAP), which is one effector of Hippo pathway (Figure 9). Thus, PrPc could be also a partner of this pathway, as shown recently for desmosomal γ-catenin in arrhythmogenic cardiomyopathy (Chen et al., 2013). PrPc interacts with YAP in both cytoplasm and nucleus, two compartments where YAP may exert opposite effects on Wnt effectors and on cell proliferation (Moroishi et al., 2015). Whether and how PrPc modulates the transcriptional activity of YAP/ or TAZ/TEAD complexes remain to be explored. Nevertheless, our results argue strongly for a role of PrPc in the regulation of gene transcription beyond the only β-catenin/TCF7L2 complex and Wnt classical targets associated to cell proliferation.

During the last ten years, several studies established a positive correlation between PrPc expression and tumor aggressiveness or adenoma to carcinoma progression (for review: (Antony et al., 2012)). Our results, which establish an interaction of PrPc with effectors of Wnt and Hippo pathways, which were both clearly involved in cancer (Clevers and Nusse, 2012; Moroishi et al., 2015), opens a new field of research on the mechanisms and the signaling pathways that link PrPc -not only its expression but also its subcellular localization and the complexes in which it is involved- to colorectal cancers.

In conclusion, PrPc is targeted toward desmosomes or nucleus in intestinal epithelial cells and shares with the armadillo family proteins, β- and γ-catenin, roles in cell-cell adhesion and cell signaling leading to the regulation of the Wnt pathway. We propose that nuclear PrPc acts as a co-regulator able to finely tune the final steps of Wnt signaling and potentially other related pathways involved in the regulation of intestinal epithelium homeostasis.

Materials and Methods
Cell culture

All culture media were purchased from Gibco/Invitrogen (Cergy-Pontoise, France). Caco-2/TC7 cells (Chantret et al., 1994) were cultured as previously described (Morel et al., 2008). SW480 and COS7 cells were cultured in high glucose DMEM Glutamax I supplemented with 10% heat inactivated fetal bovine serum (Eurobio/Abcys, Les Ulis, France) and with (SW480) or without (COS7) 1% non-essential amino acids. Non-tumoral crypt-like human intestinal cells HIEC-6, kindly provided by Dr J-F. Beaulieu, were cultured in OPTIMEM-Glutamax supplemented with 5% fetal bovine serum, HEPES 10mM (Gibco/Invitrogen) and EGF 5ng/ml (BD Biosciences, Le Pont de Claix, France). Depending on experiments, cells were plated on 3 µm pore size microporous PET filters (Transwell), on glass coverslips (Polylabo, Strasbourg, France) or on plastic (Corning Inc, Fisher, Illkirch, France).

Mass Spectrometry analysis

Proliferating Caco-2/TC7 cells were washed twice in 10 mM Tris-HCl pH 7.5 containing 20 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂ and 0.2 mM spermidine (TKCM buffer) and scrapped in TKCM containing 1% Triton X-100, 1 mM phenylmethylsulfonylfluoride (PMSF), anti-proteases and antiphosphatases. Nuclei were pelleted by centrifugation at 1000g for 10 min at 4°C, washed in TKCM buffer and nuclear proteins were extracted with 2M NaCl in TKCM buffer for 1h at 4°C. Excess NaCl was removed by overnight dialysis against PBS at 4°C. Nuclear proteins were immunoprecipitated with anti PrPc antibodies (Ab703; rabbit polyclonal antibody Abcam, Cambridge, UK) and separated on 4–12% SDS/polyacrylamide gels. After staining with colloidal Commassie blue (G250, Bio-Rad), the visualized bands were cut into slices of 1 mm. Gel slices were then reduced, alkylated and subjected to digestion with trypsin (Roche Diagnostics, Meylan, France). Extracted peptides were dried and solubilized in solvent A (95/5 water/acetonitrile in 0.1% (w/v) formic acid). The total digestion product of a gel slice was used per liquid chromatography-tandem MS (MS/MS) analysis. The extracted peptides were concentrated and separated on a LC-Packing system (Dionex S.A.) coupled to the nano-electrospray II ionisation interface of a QSTAR Pulsar i (Applied Biosystems) using a PicoTip (10 mm i.d., New Objectives, Woburn, MA). The MS/MS data was searched twice by using MASCOT (Matrix Science, London) and PHENYX (Geneva Bioinformatics S.A.) softwares on internal servers, first without taxonomic restriction to reveal the presence of proteins of interest and mammalian contaminants, then in the National Center for Biotechnology Information Human database (National Library of Medicine, Bethesda). All data are manually verified in order to minimize the errors in protein identification and/or characterization.
Immunofluorescence analyses and Proximity Ligation Assay

Cells were seeded on glass coverslips (SW480 and HIEC-6 cells) or on Transwell filters (Caco-2/TC7 cells). After 2 or 8 days, cells were fixed with paraformaldehyde (4%, 30 min, room temperature) and permeabilized with Triton X-100 (0.1%, 30 min). Alternatively, for some antibodies, cells were fixed and permeabilized with methanol (5 min, –20°C). The following antibodies were used: anti-PrP° (12F10; mouse monoclonal antibody, S.P.I. BIO; Montigny le Bretonneux, France, or Ab703; rabbit polyclonal antibody Abcam); γ-catenin and β-catenin (mouse monoclonal antibodies, BD Biosciences, Erembodegem, Belgium); γ-catenin (rabbit polyclonal antibody, Abcam); E-cadherin (ECCD2, rat monoclonal antibody, TaKaRa Bio Europe; Saint-Germain-en-Laye, France); β-catenin and TCF7L2 (rabbit polyclonal antibodies, Cell Signaling, St Quentin en Yvelines, France); FLAG (mouse monoclonal antibody, Sigma-Aldrich, St Quentin-Fallavier, France); YAP1 (rabbit monoclonal antibody, WuXi AppTec, San Diego, Ca); KI-67 (rabbit polyclonal antibody Abcam). Secondary antibodies were Alexa-488 and Alexa-546-conjugated anti-IgG (Molecular Probes, St Aubin, France).

Proximity Ligation Assay (PLA) was performed on cells processed as described above for immunofluorescence, using two primary antibodies from mouse and rabbit, and according to manufacturer’s instructions (Olink Bioscience, Sigma-Aldrich). PLA PLUS and MINUS probes for mouse and rabbit and the Duolink Orange detection kit were used.

Nuclei were stained by 4′-6-diamidino-2-phenylindole (DAPI), cells were examined by confocal microscopy (LSM 710 microscope; Carl Zeiss; Jena, Germany) using ZEN 2011 software. Quantifications were performed using a macro of Image J software (2.0.0).

Immunoprecipitation and western blots

For total protein extraction, the cell layer was washed in cold PBS and scrapped in TNE buffer (Tris 10 mM, pH 8, NaCl 150 mM, EDTA 1 mM) / Nonidet P-40 (1%) supplemented with antiproteases and antiphosphatases cocktails (Sigma-Aldrich). Alternatively, nuclear/cytoplasmic protein fractions were purified using the NE-PER Nuclear and Cytoplasmic Extraction Reagents kit-Pierce (Thermo Fischer Scientific, Illkirch, France) according to the manufacturer’s instructions.

Protein concentrations were determined using the BC Assay (Uptima/Interchim, Montluçon, France).

Immunoprecipitations were performed on nuclear extracts with rabbit anti-PrP°, anti-γ-catenin antibodies (Abcam), or non immune rabbit IgG (Sigma-Aldrich) as control, coupled to protein A-sepharose CL 4B (Amersham Biosciences, GE Healthcare, Orsay, France). For
western blots, samples were boiled for 10 minutes in Laemmli buffer and fractionated through 10 or 12% polyacrylamide gels under reducing conditions. The following antibodies were used: anti-PrP\(^c\) (mouse monoclonal SAF32; S.P.I. BIO), anti-\(\gamma\)-catenin and \(\beta\)-catenin (mouse monoclonal antibodies, BD Biosciences), anti-TCF7L2 (rabbit polyclonal antibody, Cell Signaling). Anti-actin (Millipore), poly [ADP-ribose] polymerase 1 (PARP-1), specificity protein 1 (SP1) and lactate dehydrogenase (LDH) (Santa Cruz Biotechnologies, Santa Cruz CA) antibodies were used for cell fraction purity control. Bound antibodies were detected by chemiluminescence (ECL Amersham Biosciences, GE Healthcare Europe GmbH, or ECL2 Pierce, Thermo Fischer) revealed on a Luminescence Image Analyzer (LAS-4000, Fujifilm, Courbevoie, France). Densitometric semi-quantitative analyses were performed using the Multigauge V3.0 software (Fujifilm).

Site-directed mutagenesis, transfection experiments and PLA quantifications

Mutagenesis was achieved by PCR using 50 ng of pFLAG-TCF7L2 (Idogawa et al., 2005), the high fidelity thermostable Phusion DNA polymerase (New England BIOLABS (NEB), Evry, France) and the complementary mutagenic oligonucleotides listed in supplementary Table 1. After PCR, the starting template was eliminated by DpnI digestion and the final products were used to transform competent bacteria from NEB. All selected mutants were sequenced before use.

SW480 cells were seeded on glass coverslips in 24 well plates (40 000 cells/well) and transfected 24 hours later with the different Flag-TCF7L2 constructs (500ng) using X-treme GENE HP DNA (Roche Diagnostics, Meylan France) according to the manufacturer’s instructions. PLA experiments were performed 2 days after transfection as described above using mouse monoclonal anti-FLAG combined with rabbit anti-PrP\(^c\), anti-\(\beta\)-catenin or anti-\(\gamma\)-catenin antibodies. Expression of the different constructs was analyzed by immunofluorescence with the anti-FLAG antibody in parallel wells, because PLA and immunofluorescent detection of FLAG tag could not be performed simultaneously, owing to competition of PLA probes and secondary antibodies for fixation on primary anti-FLAG antibodies.

For confocal microscopy analysis, acquisition settings were chosen so that PLA signals for \(\beta\)-catenin/FLAG interactions, which gave the highest intensities, were close to the saturation level. The same settings were then kept constant for all interactions and all constructs, maximizing the dynamic range of quantification. Quantification of PLA signals for the different Flag-TCF7L2 constructs and for the different interactions was performed using a macro of Image J software (2.0.0). Integrated intensity of PLA signal related to cell number was measured in at least 10 random fields for each condition (total of approximately 1000
cells). After subtraction of background (mean intensity obtained for PLA assays with FLAG-empty vector), values were reported to transfection efficiency of each construct, evaluated by the integrated intensity of the FLAG immunofluorescence signal related to cell number (mean of at least 10 random fields, total of approximately 1000 cells).

**Small interfering RNA (siRNA) transfection**

siRNAs were purchased from Qiagen SA Biosciences (Courtaboeuf, France). Two different siRNA sequences were combined for each gene silencing (supplementary Table 2). Cells were seeded at 20 000 cells/cm² on plastic or on glass coverslips and transfected 48h after plating using X-tremeGENE siRNA Transfection Reagent (Roche Diagnostics). The total concentration of siRNA in the media was 200 nM.

**TCF/β-catenin reporter assays**

The T-cell factor (TCF)-responsive TOP-FLASH, expressing luciferase driven by multiple TCF-responsive elements, or FOP-FLASH with mutated TCF-responsive elements, were purchased from Millipore. The pRSV-β-gal encoding β-galactosidase was used as internal control. The vectors encoding human γ-catenin and mouse PrP<sup>c</sup> were obtained from Eric Fearon (Addgene plasmid #16827)(Caca et al., 1999) and Susan Lindquist (Addgene plasmid #22109)(Jackson et al., 2009) respectively. The vector encoding a constitutively active mutant S33Y of β-catenin was obtained from Corinne Quittau-Prevostel (U1194, IRCM, Montpellier, France).

COS7 or SW480 cells were plated into 12-well plates (120 000 and 80 000 cells/well respectively). Transfection was performed 24h after plating using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics). Cells were transfected with 200 ng of TOP or FOP vector, together with 100 ng pRSV-β-gal. COS7 received in addition 50 ng S33Y β-catenin vector and either γ-catenin or PrP<sup>c</sup> vector (100 or 300 ng). SW480 received only γ-catenin or PrP<sup>c</sup> vector since β-catenin is constitutively stabilized in this cell line. The PTZ18R plasmid was used to adjust the quantity of plasmid DNA to 1 µg for all conditions. Cells were harvested 48h after transfection and β-galactosidase and luciferase activities were analyzed using a multifunctional microplate reader (FLUOSTAR Omega, BMG Labtech, Ortenberg, Germany).

TOP/FOP assays were also performed in SW480 after PrP<sup>c</sup> silencing by siRNA. In this case, reporter plasmids and the mouse PrP<sup>c</sup> expression vector were transfected 24h after plating, and siRNA were transfected the day after as described above. We showed previously that mouse PrP<sup>c</sup> was not targeted by siRNA directed against the human mRNA (Petit et al., 2012).
**Purification of epithelial cells from mouse intestinal crypts**

PrP<sup>c</sup> knockout mice, backcrossed on C57BL/6, and their wild type counterparts were housed in pathogen-free conditions (Petit et al., 2012). Ileum of three-months old wild-type (5 animals) or PrP<sup>c</sup> KO mice (5 animals) were collected, flushed with PBS containing 1mM CaCl<sub>2</sub> and 0.5 mM MgCl<sub>2</sub>, minced in one mm pieces and transferred, as a pool, in a chelation buffer (trisodium citrate 27mM, Na<sub>2</sub>HPO<sub>4</sub> 5mM, NaCl 96mM, KH<sub>2</sub>PO<sub>4</sub> 8mM, KCl 1.5mM, DTT 0.5mM, D-sorbitol 55mM, sucrose 44mM) at 4°C for shaking. According to the strength and number of agitations, 5 fractions of epithelial cells were obtained, corresponding to the villus tip (fraction 1) to the crypt bottom (fraction 5). Each cell fraction was centrifuged (1500 rpm, 5 min., 4°C); the cell pellet was resuspended in 800 µl of PBS<sup>+</sup> and stored at -80°C until RNA extraction. The analysis of the differential expression of a set of genes between the top and bottom fractions of the crypts and comparison with the results reported in (Mariadason et al., 2005) allowed attesting the purity of the bottom crypt fraction.

**RNA extraction and gene expression analyses**

Total RNA was isolated from mouse epithelial crypt cells or cultured cell lines with Tri-Reagent (Molecular Research Center, Cincinnati, OH) according to manufacturer's instructions. After rDNase digestion (Macherey-Nagel, Hoerdt, France), RNA integrity was checked by gel electrophoresis. Reverse transcription (RT) was performed with 1 µg of RNA using Roche reagents (Roche Diagnostics). Real-time PCRs were conducted with cDNA and both the sense and antisense oligonucleotides in a volume of 10µl of SYBR green PCR mix (Agilent, Massy, France) monitored and assessed in a detection system instrument (Mx3000P Stratagen). Values were normalized to L19 or 18S for human cells or to cyclophilin expression for mouse cells. Primer sequences are reported in supplementary Table 3.

**PCR array analysis**

SW480 and HIEC-6 cells were transfected with siRNA 48 hours after plating and collected 24 hours after transfection for RNA extraction as described above. After reverse transcription with RT<sup>2</sup> First strand kit using 1 µg of RNA, silencing efficiency was checked by semi-quantitative real-time PCR. A minimum of 80% extinction of the siRNA targets was a prerequisite to proceed further for PCR array analysis. Human WNT Signaling Targets RT<sup>2</sup> Profiler PCR Array plate (Qiagen SA Biosciences), which profiles the expression of 84 target genes and 8 control genes, were used with RT<sup>2</sup> SYBR Green qPCR Mastermix and the Mx3000P Stratagen system. Genes were selected for heat map profiles when modulations of their expression, after normalization by manufacturer’s controls, were above X1.4 or under
X0.7 upon either PrP\textsuperscript{c} or γ-catenin silencing in at least one cell line, with similar values in 3 independent experiments and P values <0.05.

**Immunohistochemistry on mouse intestinal crypts**

For β-catenin staining in mouse crypts, WT and PrP KO mice were euthanized, and their intestines were removed, flushed gently with PBS, and fixed overnight at 4°C in alcohol-formalin-acetic acid before embedding in paraffin. Immunostaining with anti-β-catenin (rabbit polyclonal, Cell signaling) was performed on 5-µm paraffin sections after antigen retrieval in boiling 10 mM citrate buffer (pH 6) for 10 min and permeabilization with 0.1% Triton-X100 (20 min). A horseradish peroxidase-labeled anti-rabbit antibody (Amersham Biosciences) and 3,3'-diaminobenzidine were used for revelation. β-catenin staining was examined on a Zeiss Imager-M2 microscope using ZEN 2011 software.

**Intestinal organoids**

Isolation and culture of intestinal crypts were performed as previously described (Andersson-Rolf et al., 2014), with some modifications. Briefly, the small intestine of 16-20 weeks old wild type or PrP knockout mice was isolated, cut in 5 cm long pieces and washed in cleaning solution (PBS-C: phosphate-buffered saline calcium/magnesium free, 2% Penicillin-Streptomycin, 1% Gentamycin (Gibco)). Intestinal pieces were opened longitudinally and villi were scraped off by using a coverslip. The tissue was washed by vigorous shaking in pre-cooled PBS-C and transferred into a 1 mM EDTA solution in PBS-C for 30 min at 4°C on a rotating wheel. Villi were then removed by vigorous shaking (~20 times) and the tissue was incubated at 4°C for 1h on a rotating wheel in Leibovitz medium (Sigma-Aldrich) supplemented with 5 mM EDTA, 2% Penicillin-Streptomycin, 1X Glutamax and 25 mM Hepes (Gibco). To isolate the crypts, the tissue fragments were transferred into a pre-cooled Leibovitz EDTA-free solution and vigorously shaken (~40 times). The presence of crypts was confirmed under the microscope and their number counted in a 30µL drop of crypt solution. A volume containing 300 crypts was spun down at 300 g for 5 min at 4°C, supernatant was discarded and the pellet was resuspended in 150 µL of Matrigel (Corning) half-diluted in DMEM/F12 (Gibco). Crypts were then seeded into 48-well flat-bottom plates and incubated for 15 min at 37°C. Then, Matrigel was overlaid by 300 µL of ENR medium (DMEM/F12, EGF 20 ng/mL (Peprotech, Neuilly s/ Seine, France), FGF 10 ng/mL (Peprotech), Noggin 100 ng/mL (Peprotech), Glutamax 2.5% (v/v) (Gibco), R-Spondin 500 ng/mL (R&D System), B27 1X (Gibco), N2 1X (Gibco)). The crypt number was evaluated after 6 days of culture by manual counting. Immunofluorescence staining was performed and
analyzed as described above after organoid fixation in 4% paraformaldehyde and permeabilization with Triton X-100 (0.3%, 10 min).

**Statistical analysis**

Values are expressed as mean ± s.e.m. Statistical analyses were performed using GraphPad Prism 6.0 (GraphPad Software). The statistical significance of the differences between groups was determined by Student t test, one-way or two-way ANOVA, or non-parametric Mann-Whitney or Kruskal-Wallis tests as appropriate. ANOVA and Kruskal-Wallis analyses were followed by Sidak's or Tukey's and Dunn's multiple comparisons post-tests, respectively. A value of P<.05 was considered as statistically significant.

**Acknowledgements**

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**References**


Figure 1: Nuclear localization of PrP<sup>c</sup> and γ-catenin and interaction between the two proteins in proliferating Caco-2/TC7 cells. (A) Confocal analysis of exponentially growing Caco-2/TC7 cells after immunofluorescence labeling of γ-catenin (green) and PrP<sup>c</sup> (magenta). Nuclei were stained by DAPI. Arrowheads indicate the nuclear labeling and arrows point out the junctional labeling. The merge image shows that both proteins co-localize partially in the nucleus and that PrP<sup>c</sup> is also abundant in perinuclear compartments. Bar, 20 µm. (B) Western blot analysis of nuclear (Nu) and cytoplasmic (Cyt) extracts of exponentially growing Caco-2/TC7 cells. Thirty µg of proteins were analyzed for each fraction in duplicate. PARP and LDH were used to check the purity of nuclear and cytoplasmic fractions. (C) Nuclear extracts were immunoprecipitated with anti-PrP<sup>c</sup> or anti-γ-catenin antibodies or with non-immune IgG. Immunoprecipitated fractions (IP) and nuclear extracts (input) were analyzed by western blot for γ-catenin (upper panel) or PrP<sup>c</sup> (lower panel). Molecular weight markers are shown on the right. In the lower panel, IgG are detected for IP samples even though the membrane was cut at the level of the 50kDa marker to avoid excessive trapping of the antibody. Note that the non- mono- or di-glycosylated forms of PrP<sup>c</sup> are clearly
separated on this blot (whereas they may appear as a smear on other blots); a PrP\(^c\) dimer (50 kDa) is detected also in the input line. (D) Proximity ligation assay (PLA) showing \textit{in situ} interaction between PrP\(^c\) and \(\gamma\)-catenin (white spots) in the nucleus of proliferating Caco-2/TC7 cells (left picture and enlargement of the red square) and at cell-cell contacts in confluent cells. For confluent cells, immunofluorescence labeling of E-cadherin is shown in the same field. Nuclei were stained by DAPI. Bars 10 \(\mu\)m.
**Figure 2**: Analysis of PrP<sup>c</sup>, γ-catenin, β-catenin and TCF7L2 (TCF4) levels in proliferating Caco-2/TC7, HIEC-6 and SW480 cells. (A) Western blot analysis of total protein extracts from Caco-2/TC7, HIEC-6 and SW480 cells (2 days after plating). Thirty µg of proteins were analyzed for PrP<sup>c</sup>, γ-catenin, β-catenin and TCF7L2 levels in each cell line in triplicate, actin being a loading control. Bar graphs show the ratio of each protein to actin after densitometric analyses (mean ± s.e.m.; *P<.05, **P<.01 ***P<.001 vs Caco-2/TC7; $P<.05, $$$P<.001$ vs SW480). (B) Western blot analysis of nuclear (Nu) and cytoplasmic (Cyt) extracts of the three cell lines. Thirty µg of proteins were analyzed for PrP<sup>c</sup>, γ-catenin and β-catenin levels in each fraction and each cell line. Purity of nuclear and cytoplasmic fractions was checked by SP1 and LDH analyses; * indicates a non-specific band revealed by the anti-SP1 antibody.
Figure 3: Subcellular distribution of PrPc, β-catenin, γ-catenin and TCF7L2 in proliferating Caco-2/TC7, SW480 and HIEC-6 cells. Cells were immunolabeled for PrPc and γ-catenin (three upper panels), PrPc and β-catenin (three middle panels), or PrPc and TCF7L2 (three bottom panels) and analyzed by confocal microscopy. Merge images with DAPI staining are shown for each labeling. Arrowheads show the faint nuclear labeling of all proteins in HIEC-6 cells and arrows point out junctional labeling of PrPc, β-catenin and γ-catenin in Caco-2/TC7 cells. Bars, 20 µm.
Figure 4

**Figure 4**: PrP<sup>c</sup> interacts with γ-catenin and with the Wnt pathway effectors β-catenin and TCF7L2 in Caco-2/TC7, SW480 and HIEC-6 cells. Proximity ligation assay (PLA) showing in
*situ* interaction (white spots) between PrP\(^{\circ}\), γ-catenin, β-catenin and TCF7L2 in proliferating cells for each cell line. PLA assays were performed between the couple of proteins indicated on the left. Nuclei were stained by DAPI. Note that interactions are detected mainly in the nucleus for Caco-2/TC7 and SW480 cells and mainly in the cytoplasm for HIEC-6 cells. Bar, 10 µm.
**Figure 5**: PrP<sup>c</sup> interaction with the transcription factor TCF7L2 occurs mainly via the β-catenin binding domain of TCF7L2. (A) Schematic diagram of the human TCF7L2 protein and the full length TCF7L2 tagged with the FLAG octapeptide (FLAG-TCF7L2<sub>FL</sub>) construct. SW480 cells were transfected with FLAG-TCF7L2<sub>FL</sub>. Transfected cells were immunolabeled using an anti-FLAG antibody (IF FLAG) and PLA were performed using anti-FLAG and anti-β-catenin (PLA β-cat/FLAG), anti-γ-catenin (PLA γ-cat/FLAG) or anti-PrP<sup>c</sup> antibodies (PLA PrP<sup>c</sup>/FLAG). Nuclei were stained by DAPI (Bar, 20 µm). Graphs present the quantifications of PLA signal for each interaction reported to the transfection efficiency (IF FLAG signal), measured in at least 10 random fields (approximately 1000 cells per experiment) in two independent experiments (A.U. arbitrary units; mean ± s.e.m.; *** P<.001 vs PLA β-cat/FLAG). NLS, nuclear localization signal; DBD, DNA binding domain. (B) Schematic diagram of the different FLAG-TCF7L2 deletion constructs: TCF7L2 lacking the β-catenin interaction domain (TCF7L2<sub>del 1-51</sub>), lacking both β- and γ-catenin interaction domains (TCF7L2<sub>del 2-100</sub>), lacking the 52-82 γ-catenin interaction domain (TCF7L2<sub>del 52-143</sub>) or deleted
for a sequence adjacent to the γ-catenin interaction domain (TCF7L2\(^{\text{del } 82-143}\)). Experiments were conducted as in (A). Graphs present the quantifications of PLA signal reported to the transfection efficiency of each construct (IF FLAG signal). Measurements were performed as described in (A). For each interaction, results obtained with the different constructs are presented as % of the value obtained with the full-length (FLAG-TCF7L2\(^{FL}\)) construct (mean ± s.e.m.; ** P<.01 and *** P<.001 vs FL).
**Figure 6**: Impact of β-catenin or γ-catenin silencing on PrPc/TCF7L2 interactions. SW480 were transfected with the indicated siRNA and analyzed after 48h. (A) Decrease of β-catenin or γ-catenin protein levels was evaluated by immunofluorescence labeling (IF). Graphs present the quantification of total (white bars) or nuclear (hatched bars) IF signal, measured in 5 random fields (approximately 300 cells; mean ± s.e.m.; $ P<.05$ and $$P<.01$ vs siCtl for total signal). (B) Evaluation by PLA of the interactions of PrPc with TCF7L2, β-catenin or γ-catenin. Graphs present the quantification of total (white bars) or nuclear (hatched bars) PLA signal per cell measured in at least 10 random fields (approximately 800 cells; mean ± s.e.m.; $ P<.05$ and $$P<.001$ vs siCtl for total PLA signal; ** $ P<.01$ vs siCtl for nuclear signal).
Figure 7: The TCF7L2/β-catenin transcriptional activity is up-regulated by PrP\(^c\) and down-regulated by γ-catenin. (A) COS7 were transfected with luciferase reporter plasmids containing TCF binding sites (TOP) or mutated TCF binding sites as negative control (FOP) and a LacZ expression plasmid as internal control. Expression vectors for constitutively active S33Y mutant of β-catenin (β-cat\(^*\)), PrP\(^c\), or γ-catenin were co-transfected as indicated. Values represent mean ± sem of luciferase activity normalized to corresponding β-Gal activity. **P<0.01 ***P<0.001 vs β-cat\(^*\) alone. (B) SW480 cells were transfected with the TOP/FOP reporters and LacZ plasmid and co-transfected with empty vector (control) or PrP\(^c\) or γ-catenin expression plasmids as indicated. **P<0.01 ***P<0.001 vs control. (C) SW480 cells were transfected with the TOP/FOP reporters and LacZ plasmid, together with a control siRNA (siCtl), a PrP\(^c\) siRNA (siPrP) or a PrP\(^c\) siRNA combined with a mouse PrP\(^c\) expression vector (siPrP+PrP\(^c\)). **P<0.01 ***P<0.001 vs siCtl; $$$ P<0.001 vs siPrP. Experiments were all performed in triplicates and the graphs present one experiment representative of two or three independent experiments for each condition.
Figure 8: PrPc and γ-catenin silencing impact on Wnt target gene expression with different effects in SW480 and HIEC-6 cells. (A) PrPc and γ-catenin knockdown in SW480 and HIEC-6 cells upon siRNA transfection. Decrease of PrPc or γ-catenin mRNA 24h after transfection with the corresponding couples of siRNAs. mRNA levels were determined by RT-qPCR and normalized to L19 levels. For each cell line, results are reported to mRNA levels in cells transfected with control siRNA, which was set at 1 (mean ± s.e.m. from 3 independent experiments; *** P<.001 vs cells transfected with control siRNA). (B) Heat maps illustrating the modifications of Wnt target gene expression 24h after PrPc or γ-catenin siRNA transfection in each cell line (analysis of 84 Wnt target genes). Genes whose expression was modulated (<0.7 fold or >1.4 fold) in at least one cell line by one siRNA were selected (mean of n=3 independent experiments for each siRNA in each cell line; P<.05 for each gene in each cell-line as compared to control siRNA). Genes were ranked according to the modulation factor of their expression in SW480 cells upon PrPc silencing. Grey color indicates undetected expressions. Note that the TCF4 modulated gene appearing in the heat map (also known as ITF2 or SEF2) differs from TCF7L2 (widely known as TCF4). (C)
Pseudo heat map after selection of the genes whose expression is increased above 1.4 fold (red) or decreased beyond 0.7 fold (green) after PrP<sup>c</sup> silencing in each cell line. Black: modulation factor comprised between 0.7 and 1.4; Grey: undetected expressions. (D) Analysis of several Wnt target genes in the fraction of epithelial cells corresponding to the crypt bottom of wild type (WT) and PrP knockout (PrP KO) mice (pool of 5 mice for each genotype). mRNA levels were determined by RT-qPCR and normalized to cyclophilin levels. Results are expressed as the ratio of expression in PrP<sup>c</sup> knockout versus WT mice for each gene.
**Figure 9**: PrP\(^c\) interacts with YAP, the effector of the Hippo pathway. Proximity ligation assays (PLA) were performed in proliferating Caco-2/TC7, SW480 and HIEC-6 cells to reveal interactions between endogenous YAP and PrP\(^c\). Note that, although present also in the cytoplasm, interaction signals were concentrated in the nucleus, even for HIEC-6 cells. Bar, 10 µm.
Figure 10: Formation of intestinal organoids is impaired in the absence of PrP<sup>c</sup>. (A) Organoid initiation frequency from intestinal crypts of WT or PrP KO mice. Each symbol (■, ○, ▲) is the mean number of organoids per well 6 days after seeding (2 to 6 wells per experiment) expressed as % of plated crypts. Bars represent the mean ± s.e.m. from the 3 independent experiments. (B) Phase contrast microscopy images 7 days after plating, showing the smaller size of organoids obtained from PrP KO mice. Bar, 200 µm. (C) E-cadherin, β-catenin and KI-67 immunostaining of 7-days organoids. Although smaller and less abundant, PrP KO organoids have a normal epithelial organization, as shown by E-cadherin and β-catenin staining (upper panels), but exhibit smaller KI-67 positive crypt domains (lower panels). Note that using the same focus for both genotypes, only part of the WT organoids may be visualized in the fields. Bar, 50 µm.
Figure 11: Altered nuclear β-catenin staining in the intestinal crypts of PrP knockout mice. (A) Crypt section of jejunum from WT and PrP knockout mice were stained for β-catenin by immunohistochemistry. Arrows indicate examples of nuclear β-catenin and arrowheads point out examples of diffuse and/or membranous staining. Bar, 10µm. (B) Quantification of cells per crypt in which β-catenin was localized mainly in the nucleus. 34 crypts from 5 WT mice and 55 crypts from 5 PrP KO mice were quantified. Whiskers mark 10th and 90th percentiles, boxes mark 25th and 75th percentiles and the black circle represents an outlier. ***P<0.001 (Student t test).
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<td>Glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>SCCA2/SCCA1 fusion protein isoform 1</td>
<td>gi 33317676</td>
<td>44648</td>
<td>4</td>
<td>12.6</td>
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**Table 1**: Proteomic analysis of nuclear PrP<sup>c</sup> partners. Nuclear extracts from proliferative Caco-2/TC7 cells (2 days) were immunoprecipitated with anti PrP<sup>c</sup> antibodies. The presence of PrP<sup>c</sup> in the resulting material was checked by western blot before identification of the interacting proteins by liquid chromatography-tandem MS (MS/MS). The number of peptide matches that was obtained after trypsination for each protein as well as the accession number (NCBI) and the molecular weight (Mr) are reported.