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SHORT REPORT

WNT/β-catenin signaling inhibits CBP-mediated RelA acetylation and expression of proinflammatory NF-κB target genes

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ABSTRACT

The discovery of functional crosstalk between WNT and nuclear factor κB (NF-κB) signaling has established a more complex role for these two pathways in inflammation and cancer. However, the molecular mechanisms of the crosstalk and its biological consequences are largely unknown. Here, we show that WNT/β-catenin signaling selectively inhibits the expression of a proinflammatory subset of IL-1β-induced NF-κB target genes. WNT/β-catenin signaling does not affect nuclear translocation of the RelA subunit of NF-κB or its association with CBP (also known as CREBBP), but reduces CBP-mediated acetylation and chromatin recruitment of RelA. Thus, β-catenin selectively regulates NF-κB gene expression through its negative effects on RelA acetylation. This anti-inflammatory effect may be relevant for cancer treatment.

KEY WORDS: Acetylation, β-catenin, CBP, NF-κB, WNT

INTRODUCTION

The inducible transcription factor nuclear factor κB (NF-κB) is crucial for inflammation, immunity and tissue homeostasis (Lawrence, 2009; Li and Verma, 2002; Pasparakis, 2009), and appears to be a crucial link between inflammation and cancer (Ben-Neriah and Karin, 2011; Karin, 2009). cAMP-responsive-element-binding protein (CREB)-binding protein (CBP, also known as CREBBP) and p300 (also known as EP300) are co-activators of NF-κB (Gerritsen et al., 1997; Perkins et al., 1997). CBP- and p300-mediated acetylation of NF-κB alters its biological activity, such as its DNA-binding affinity, transcriptional activity and interaction with other proteins (Chen et al., 2002; Furia et al., 2002). Importantly, acetylation of the RelA subunit of NF-κB at K310 and K314 by CBP and/or p300 regulates the expression of a subset of NF-κB-dependent genes (Chen et al., 2002; Rothgiesser et al., 2010a,b).

WNTs are secreted signaling proteins involved in embryonic development and homeostatic self-renewal of adult tissues. Upon activation by WNTs, β-catenin translocates to the nucleus, where it associates with TCF/LEF family transcription factors to activate target gene expression. CBP and p300 transcriptionally co-regulate canonical WNT signaling (Hecht et al., 2000; Takemaru and Moon, 2000). Although WNT/β-catenin has been ascribed tumor-stimulating effects, it also seems to mediate anti-inflammatory effects (Deng et al., 2002; Kim et al., 2005; Ma et al., 2012). However, the molecular mechanism by which this occurs and how the specificity is regulated is unknown. Here, we show that WNT/β-catenin negatively regulates the expression of a proinflammatory subset of NF-κB target genes in interlekin-1β (IL-1β)-stimulated human lung fibroblast and carcinoma cells by inhibiting the CBP-mediated acetylation of RelA.

RESULTS AND DISCUSSION

WNT/β-catenin signaling selectively reduces IL-1β-induced NF-κB target gene expression

WNT/β-catenin signaling does not seem to regulate all NF-κB target genes (Kim et al., 2005). To study the mechanism of this selective repression, we used primary human MRC-5 lung fibroblasts, which are responsive to both NF-κB and WNT pathway activation (Baarsma et al., 2011; Sfikas et al., 2012). Treatment with WNT-3A increased β-catenin protein levels (supplementary material Fig. S1A) and expression of the target gene *AXIN2* (supplementary material Fig. S1B), indicating activation of canonical WNT/β-catenin signaling in these cells. WNT-3A treatment significantly decreased IL-1β-induced *IL1B*, *IL6* and *CSF2* expression, but not *ICAM1* or *VCAM1* expression (Fig. 1A; supplementary material Fig. S1B). The negative effect increased with time and correlated with higher β-catenin levels. Identical results were obtained in human IMR-90 lung fibroblasts and A549 lung carcinoma cells (supplementary material Fig. S1C,D). In MRC-5 cells, WNT-3A inhibited expression of the same set of genes also when stimulated with TNFα (supplementary material Fig. S2), suggesting that WNT/β-catenin represses the expression of a subset of NF-κB-driven target genes regardless of the stimulus and the cell type. RelA knockdown confirmed that all tested target genes were downstream of NF-κB (Fig. 1B; supplementary material Fig. S3A). Overexpression of β-catenin had the same selective negative effect on *IL1B* and *IL6* (supplementary material Fig. S3B; Fig. 1C). By contrast, knockdown of β-catenin enhanced IL-1β-induced expression of *IL1B* and *IL6*, regardless of whether the cells were stimulated with WNT-3A or not (Fig. 1D; supplementary material Fig. S3C). Importantly, knockdown of the canonical WNT pathway transcription factor TCF4 did not eliminate the WNT-3A effect, whereas knockdown of TCF4 or β-catenin significantly reduced expression of the WNT target gene *AXIN2* (Fig. 1D; supplementary material Fig. S3C), demonstrating that β-catenin mediates the selective negative effect of WNT signaling on NF-κB target gene expression.

CBP regulates IL-1β-induced target gene expression and mediates the negative effects of WNT-3A

Although CBP knockdown attenuated IL-1β-induced *IL1B*, *IL6* and *CSF2*, and not *ICAM1* or *VCAM1* expression (Fig. 2A; supplementary material Fig. S3D), this was not observed for p300 (Fig. 2B; supplementary material Fig. S3E). Furthermore, depletion of CBP eliminated the negative effect of WNT-3A on IL-1β-induced *IL1B* and *IL6* expression (Fig. 2C), strongly suggesting that CBP, but not p300, mediates the effect of WNT/β-catenin on NF-κB in MRC5 cells and is involved in the crosstalk between WNT and NF-κB signaling.

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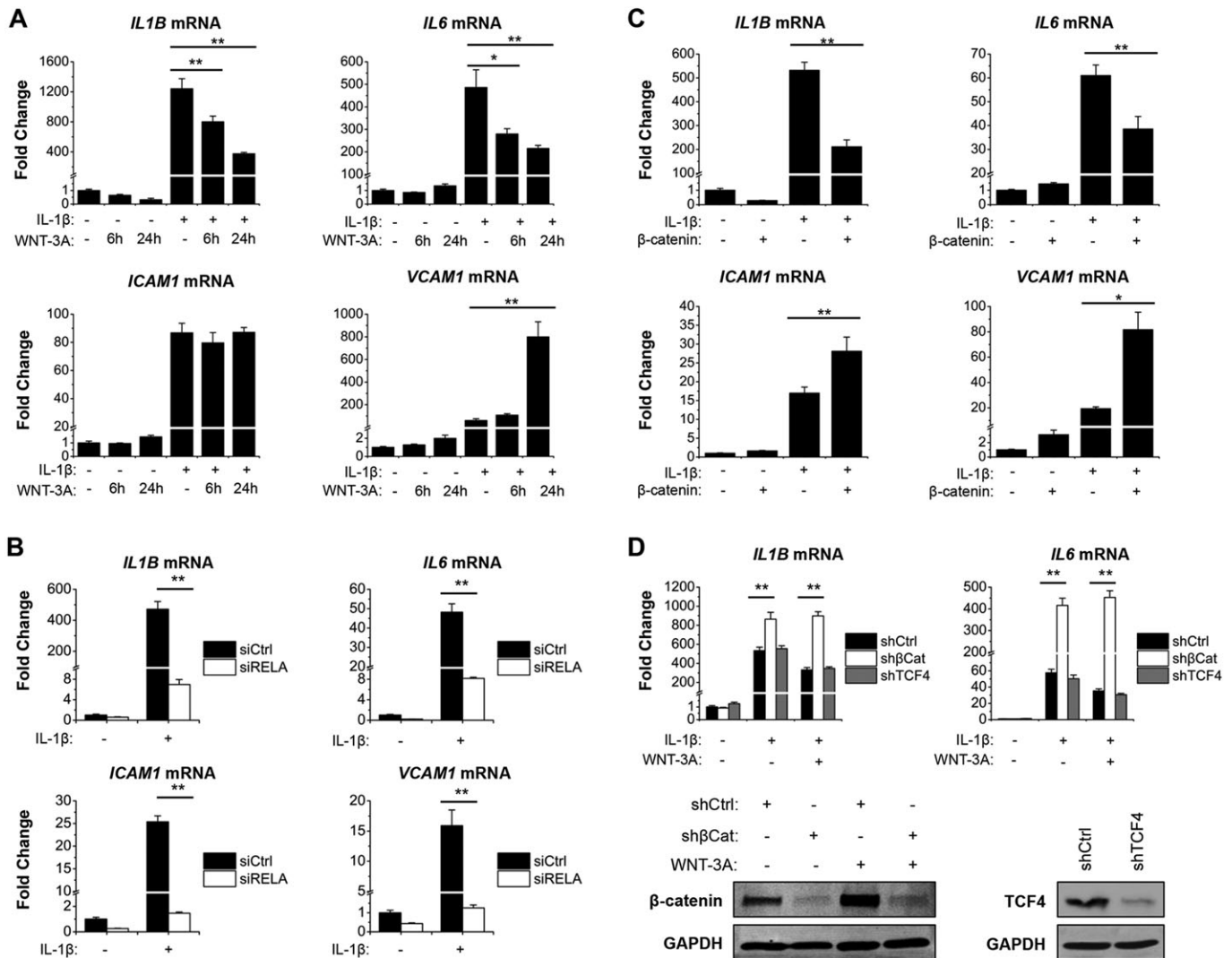
Fig. 1. WNT/β-catenin signaling selectively reduces IL-1β-induced NF-κB target gene expression. (A) MRC-5 cells were treated with WNT-3A for the indicated times and IL-1β was added 4 h before harvest of samples. mRNA expression was measured by qRT-PCR. *P<0.05; **P<0.01; n=3. (B) MRC-5 cells were transfected with control siRNA (siCtrl) or siRNA against RelA (siRELA) and then stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. **P<0.01; n=3. (C) MRC-5 cells transduced with lentiviruses expressing empty control or β-catenin were stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. *P<0.05; **P<0.01; n=3. (D) MRC-5 cells were transduced with lentiviruses expressing control shRNA (shCtrl), or shRNA against β-catenin (CTNNB1) (shβCat) or TCF4 (shTCF4) for 3 days and then stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. **P<0.01; n=3. Protein expression was analyzed by western blotting (a representative blot of two independent experiments is shown).

WNT/β-catenin signaling inhibits CBP-mediated acetylation of RelA

IL-1β-induced nuclear translocation of RelA was not affected by WNT-3A treatment as judged by immunofluorescence (Fig. 3A) and western blotting of nuclear and cytoplasmic extracts (Fig. 3B). Depletion of CBP has been shown to attenuate the interaction between RelA and β-catenin (Schwitalla et al., 2013). However, it is unclear whether RelA and β-catenin interact through CBP only, or whether other proteins such as E-cadherin are also involved (Solanas et al., 2008). We cannot exclude the possibility that RelA and β-catenin already directly interact with each other in the cytoplasmic compartment (Fig. 3A). Although IκB-α (also known as NFKBIA) was found in complex with RelA, we could not demonstrate that β-catenin directly interacted with RelA in pulldown experiments using recombinant proteins (supplementary material Fig. S4A). Immunoprecipitation of CBP in nuclear extracts revealed that RelA and β-catenin are both bound to CBP and that

this interaction was unaffected by WNT-3A stimulation (Fig. 3C), although we cannot exclude that RelA and β-catenin bind to separate CBP molecules. Experiments with recombinant proteins showed that RelA and β-catenin both strongly associated with CBP (supplementary material Fig. S4B). Moreover, immunoprecipitation of RelA in nuclear extracts revealed that CBP and β-catenin were both bound to RelA, and binding of CBP to RelA was unaffected by WNT-3A treatment (Fig. 3D), indicating that β-catenin does not compete with RelA for binding to CBP, but that β-catenin might inhibit RelA activity indirectly by binding to CBP.

Although IL-1β induced acetylation only at K314, whereas acetylation of K310 was stimulus-independent in the tested cells, acetylation of RelA at K314 was decreased by WNT-3A co-stimulation or overexpression of β-catenin (Fig. 3E,F). Acetylation of RelA at K314 was only observed in the nuclear fraction and was hardly detected in the cytoplasmic fraction (supplementary material
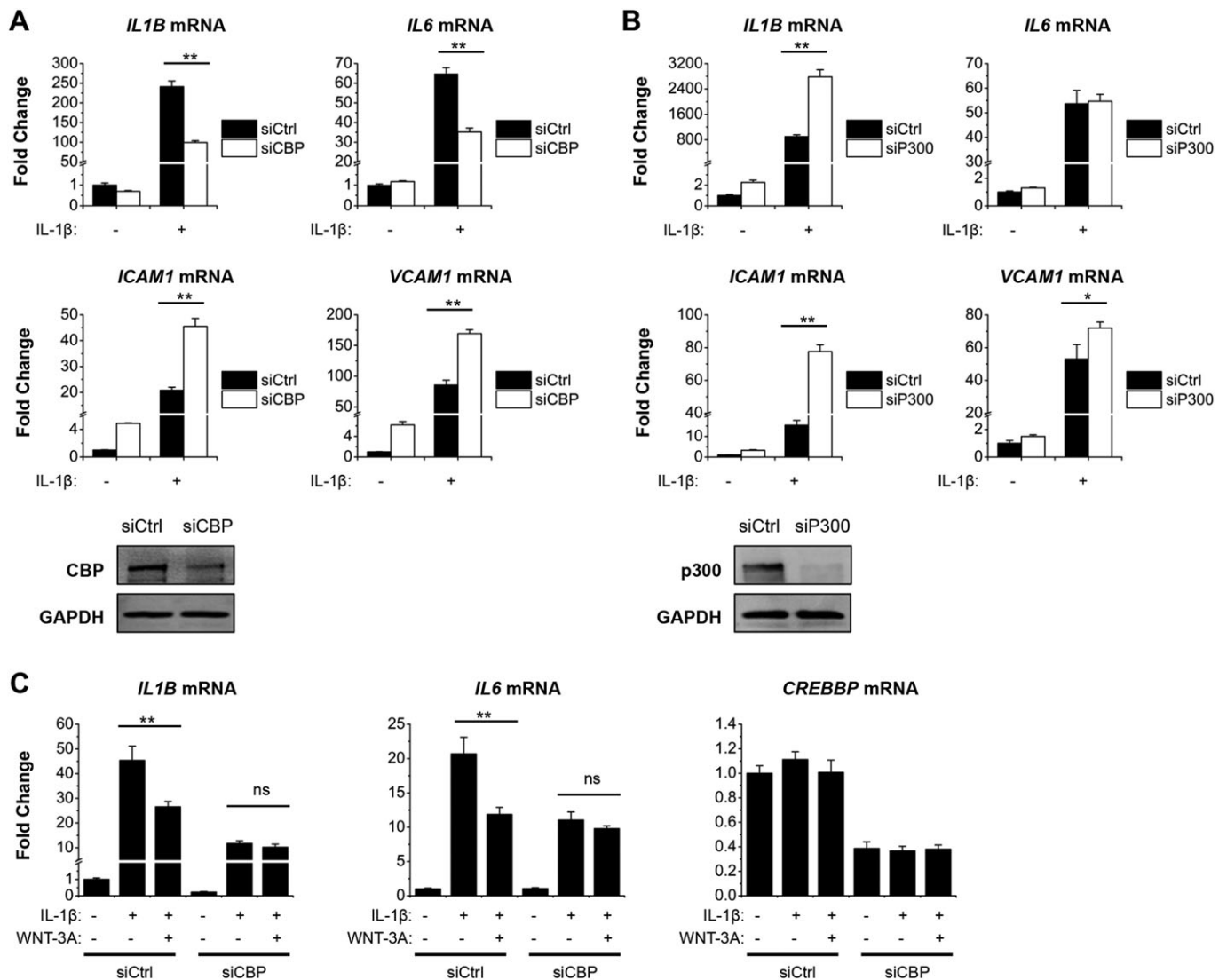
Fig. 2. CBP regulates IL-1β-induced target gene expression and mediates the negative effects of WNT-3A. (A) MRC-5 cells were depleted of CBP (CREBBP) by siRNA tranfection and stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. **$P<0.01$; $n=3$. Protein expression was analyzed by western blotting. (B) MRC-5 cells were depleted of p300 (EP300) by siRNA transfection and stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. *$P<0.05$; **$P<0.01$; $n=3$. Protein expression was analyzed by western blotting (a representative blot of two independent experiments is shown). (C) CBP depletion attenuated the negative effects of WNT-3A on IL-1β-induced NF-κB target gene expression. MRC-5 cells were transfected with siRNA and then stimulated with WNT-3A for 24 h and IL-1β was added 4 h before harvest of samples. mRNA expression was measured by qRT-PCR. **$P<0.01$; ns, not significant; $n=3$.

Fig. S4C), indicating that acetylation of RelA by CBP is a nuclear event. CBP knockdown confirmed that the observed RelA acetylation at K310 and K314 *in vivo* was mediated by CBP (Fig. 3G). In contrast, knockdown of p300 did not influence IL-1β-induced acetylation of RelA K314 (supplementary material Fig. S4D), suggesting that CBP is the predominant acetyltransferase for this site in MRC-5 cells. β-Catenin reduced CBP-mediated acetylation of RelA at K310 and K314 *in vitro* (Fig. 3H). Thus, our data show that β-catenin directly inhibits RelA acetylation by CBP and not the association of CBP with RelA.

To directly test the function of K314 acetylation, wild-type or K314R mutated Myc-tagged RelA was overexpressed in MRC-5 cells (supplementary material Fig. S4E). Overexpression of K314R RelA caused less induction of *IL1B* and *IL6* mRNA expression, whereas upregulation of *ICAM1* and *VCAM1* expression was not different when compared to overexpression of wild-type RelA (Fig. 3I; supplementary material Fig. S4E), implying that K314 acetylation

is important for the expression of a subset of NF-κB target genes. Decreased K314 acetylation might therefore be responsible, at least partially, for the negative effect of WNT/β-catenin on a subset of NF-κB target genes.

WNT/β-catenin activation reduces DNA binding activity of RelA

The duration of nuclear NF-κB induction is regulated by reversible acetylation (Chen et al., 2001). We therefore assessed chromatin recruitment of RelA and CBP to target genes. Chromatin immunoprecipitation (ChIP) experiments confirmed that both RelA and CBP bound to the promoters of *IL1B* and *IL6* and that their recruitment was inhibited by WNT-3A co-treatment (Fig. 4A). Binding of RelA or CBP to the *ICAM1* or *VCMA1* promoter was not significantly altered (Fig. 4A; supplementary material Fig. S4F), in line with *ICAM1* and *VCAM1* mRNA expression not being decreased by WNT/β-catenin. In contrast to β-catenin binding to
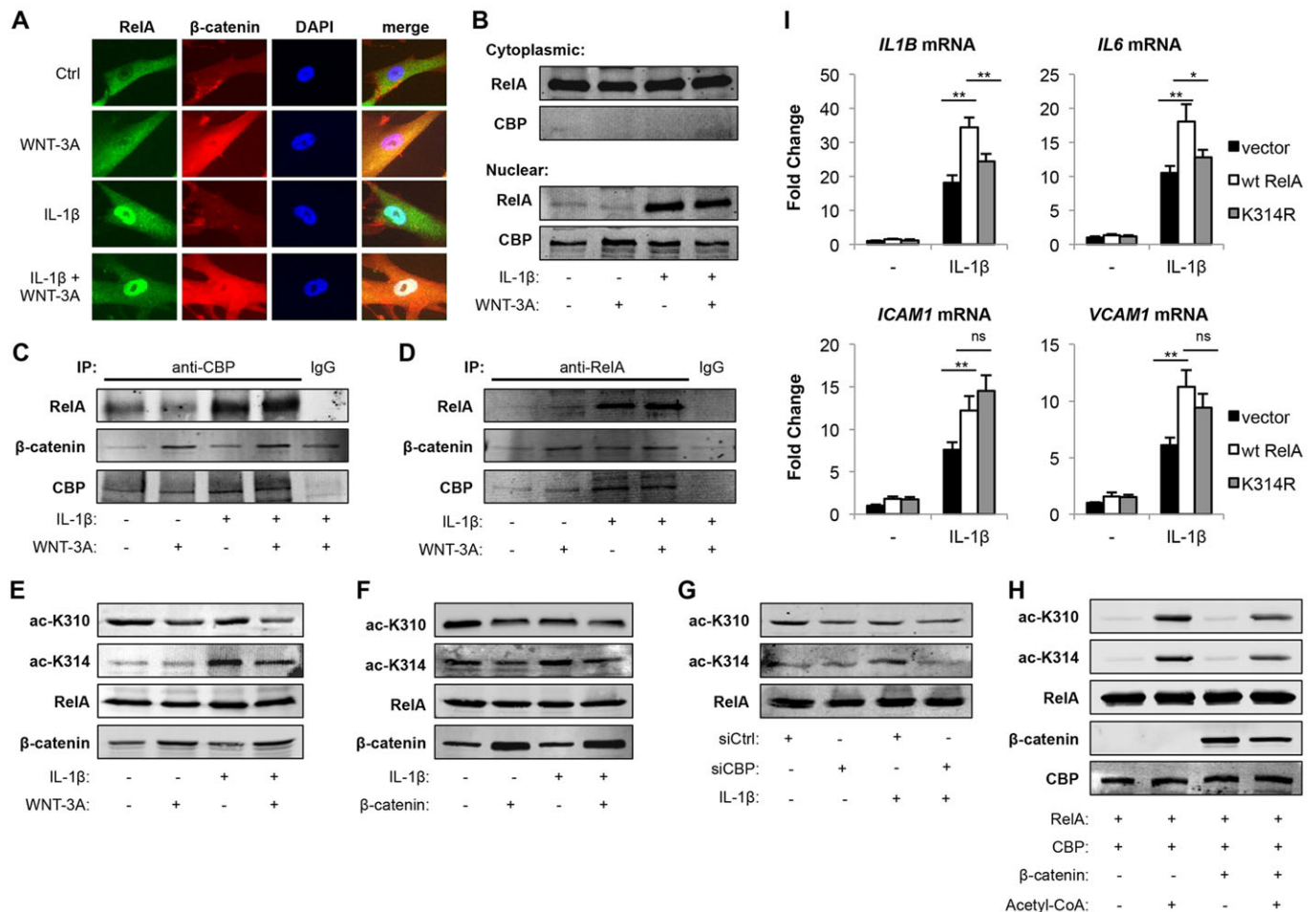
Fig. 3. WNT/β-catenin signaling inhibits CBP-mediated acetylation of RelA. (A) Immunofluorescence for RelA (green), β-catenin (red) and the nucleus (DAPI, blue) in MRC-5 cells stimulated with WNT-3A for 18 h and IL-1β added 4 h before harvest of samples. (B) Western blot analysis of RelA and β-catenin in the nuclear and cytoplasmic fraction of MRC-5 cells stimulated with WNT-3A for 18 h, with IL-1β added 1 h before harvest of samples as indicated. (C,D) MRC-5 cells were stimulated with WNT-3A for 18 h and IL-1β was added 1 h before harvest of samples. Nuclear proteins of MRC-5 were immunoprecipitated (IP) with anti-CBP (C) or anti-RelA (D) antibodies and then subjected to western blot analysis. IgG served as control for non-specific binding. (E) MRC-5 cells stimulated with WNT-3A for 18 h, with IL-1β added 30 min before harvest of samples as indicated. Acetylated RelA at K310 (ac-K310) and K314 (ac-K314) was analyzed by western blotting. (F) MRC-5 cells were transduced with lentiviruses expressing empty control or β-catenin and then stimulated with IL-1β for 1 h. Total protein lysates were then subjected to western blot analysis. (G) MRC-5 cells were transfected with control siRNA (siCtrl) or siRNA against CBP (siCBP) and then stimulated with IL-1β for 30 min. Total protein lysates were subsequently subjected to western blot analysis. (H) β-catenin inhibits *in vitro* acetylation of RelA. Proteins were subjected to western blot analysis after incubation of RelA with CBP in the presence or absence of β-catenin or acetyl-CoA. (I) MRC-5 cells were transfected with empty vector, wild-type (wt) RelA or K314R mutant plasmids and then stimulated with IL-1β for 4 h. mRNA expression was measured by qRT-PCR. *P<0.05; **P<0.01; ns, not significant; *n*=3. For western blot data, representative blots of two independent experiments are shown.

the *KAI1* promoter through the p50 NF-κB subunit (Kim et al., 2005), we did not observe any binding of β-catenin to the *IL1B* or *IL6* promoter. Increased β-catenin binding was found to the *AXIN2* promoter (supplementary material Fig. S4F). These data indicate that β-catenin regulates the NF-κB target genes only through binding to CBP and not by another mechanism (e.g. by inducing chromatin remodeling). Interestingly, increased binding of CBP and β-catenin to the *VCAM1* promoter was also observed (supplementary material Fig. S4F), which might explain the synergistic effect of WNT and NF-κB activation on *VCAM1* expression, revealing a more complex regulatory mechanism. To confirm that impaired RelA acetylation by β-catenin is responsible for reduced NF-κB chromatin recruitment, we performed ChIP assays using an antibody specific for RelA acetylated at K314. The latter was recruited to the *IL1B* and *IL6* promoters and this recruitment was inhibited by WNT-3A treatment (Fig. 4B). Although gene expression of *ICAM1* and *VCAM1*, or binding of

total RelA to their promoters, was not significantly decreased by WNT-3A signaling, a decrease in the recruitment of K314 acetylated RelA to these promoters was observed (Fig. 4B; supplementary material Fig. S4G), suggesting that IL-1β-induced transcription of *ICAM1* or *VCAM1* does not require RelA K314 acetylation. This is supported by the fact that induction of the expression of the two genes in cells overexpressing K314R RelA was the same as with wild-type RelA (Fig. 3I), and is consistent with previous findings showing that chromatin-associated RelA is acetylated at K314 on genes whose expression is not affected by mutating K314 (Rothgiesser et al., 2010b). These results indicate that the dependency of the transcriptional activation of genes on the RelA acetylation status is gene specific.

In conclusion, we show that WNT/β-catenin signaling selectively suppresses the expression of a proinflammatory subset of NF-κB target genes. Decreased enzymatic activity of CBP, and not decreased nuclear translocation of RelA or binding of CBP to
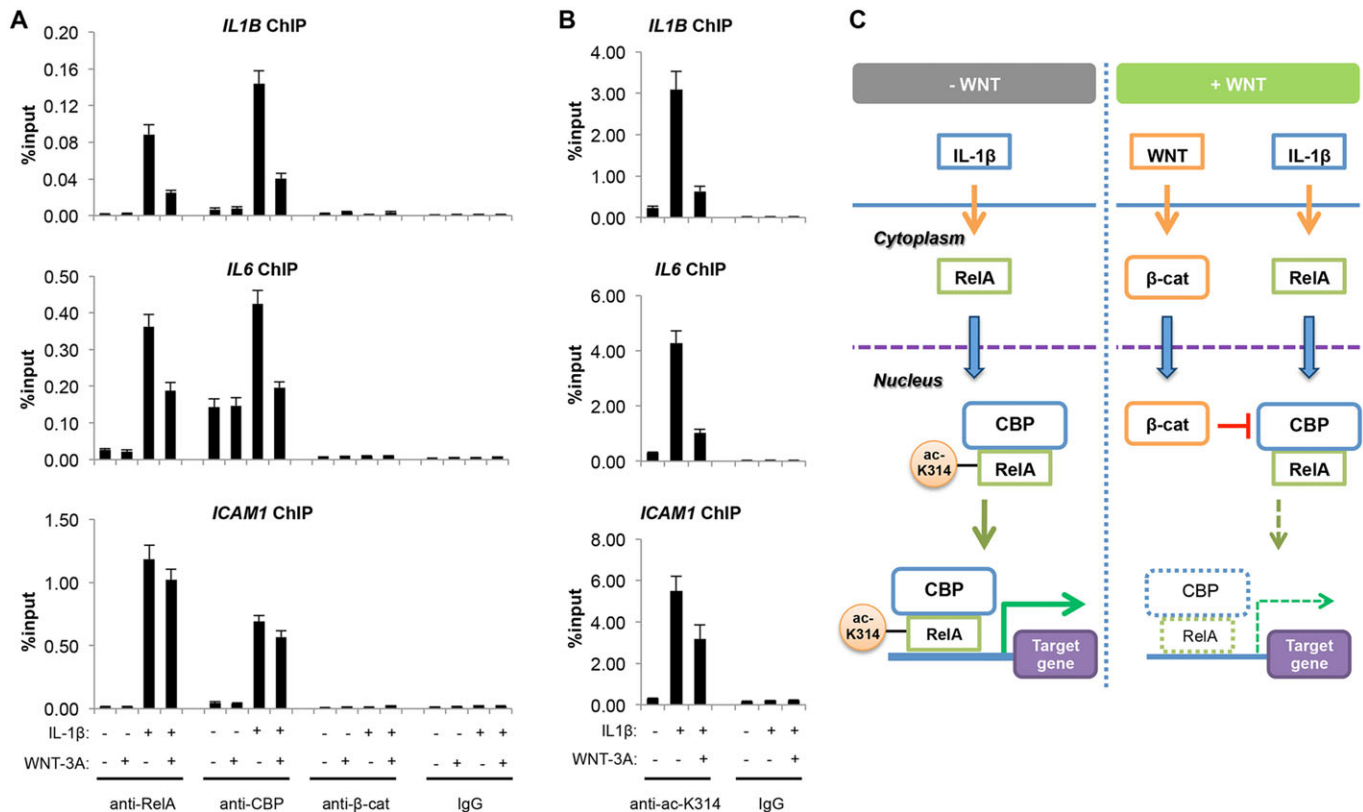
Fig. 4. WNT/β-catenin activation reduces the DNA-binding activity of RelA. (A) MRC-5 cells were stimulated with WNT-3A for 18 h and IL-1β was added 1 h before harvest of samples. Binding of RelA, CBP or β-catenin to NF-κB target gene promoters was analyzed by ChIP. (B) MRC-5 cells were stimulated with WNT-3A for 18 h and IL-1β was added 1 h before harvest of samples. Binding of K314-acetylated RelA to NF-κB target gene promoters was analyzed by ChIP. (C) Working model of the mechanism by which WNT/β-catenin signaling inhibits expression of proinflammatory NF-κB target genes.

RelA, is responsible for this effect. Thus, β-catenin induced by WNT stimulation attenuates CBP-mediated acetylation of RelA K314 induced by IL-1β, reducing its recruitment to chromatin and the expression of a subset of NF-κB target genes (Fig. 4C). Although enhanced β-catenin levels did not cause a loss of interaction between CBP and RelA, implying that the interaction of RelA to CBP is not dependent on RelA acetylation, β-catenin reduced recruitment of both RelA and CBP to the chromatin. This suggests that the interaction of β-catenin with CBP affects the ability of the CBP–RelA complex to interact with chromatin through acetylation of RelA. The detailed molecular mechanism of how β-catenin affects CBP-mediated RelA acetylation remains to be elucidated. A possible mechanism might be through the induction of a specific conformational change in CBP that does not affect binding to RelA. Our data have uncovered an important anti-inflammatory mechanism of canonical WNT signaling and β-catenin. Increased NF-κB activity due to depletion of β-catenin in dendritic cells might explain the observed elevated inflammatory response and disease severity in a mouse model of inflammatory bowel disease (Manicassamy et al., 2010). A recent study shows that induced NF-κB might participate in β-catenin-mediated target gene expression and eventually results in enhanced tumor growth (Schwitalla et al., 2013), adding to the tumorigenic mechanisms of NF-κB-mediated inflammation. Whether acetylated RelA influences WNT/β-catenin target gene expression remains to be answered. One is tempted to speculate that blockage of the WNT pathway, leading to decreased β-catenin, might result in enhanced NF-κB signaling and consequently inflammation-triggered tumorigenesis.

MATERIALS AND METHODS

Reagents and antibodies

Human IL-1β and TNFα were from Sigma-Aldrich, WNT-3A (high purity) was from R&D Systems, and anti-p65/RelA (A), anti-β-catenin (H-102), anti-TCF4 (D-4), anti-CBP (A-22), anti-p300 (sc-585) anti-IκB-α (C-21) and anti-GAPDH (FL335) antibodies and normal IgG were from Santa Cruz Biotechnologies. The acetyl-specific antibodies for anti-acetyl K310 (ab19870) and anti-acetyl K314 (ab18727) p65/RelA and anti-Myc tag antibody (ab9132) were from Abcam.

siRNAs and plasmids

siRNAs against human RelA (*RELA*), CBP (*CREBBP*, two different sequences), p300 (*EP300*, three different sequences) and non-silencing control were from Qiagen. The lentiviral expression vector for human β-catenin was generated by transferring β-catenin cDNA into pLenti-CMV-Puro-DEST vector (Addgene plasmid 17452) through pENTR4 (Addgene plasmid 17424) (Campeau et al., 2009) using the LR Clonase™ II enzyme mix (Life Technologies). The following lentiviral small hairpin RNA (shRNA) expression vectors were used: pLKO.1-shCtrl (control shRNA) (Addgene plasmid 1864) (Sarbassov et al., 2005), pLKO.1-shβ-Cat (shRNA against β-catenin) (Addgene plasmid 18803) (Onder et al., 2008) and pLKO.1-shTCF4 (shRNA against TCF4) (Ma et al., 2012).

Generation of recombinant proteins

Human RelA, CBP and IκB-α were generated by baculovirus expression. Human β-catenin was generated by using the pGEX GST gene fusion system.

Cell culture, transfection and viral transduction

MRC-5 and IMR-90 (ATCC) were cultured in minimal essential medium (MEM), A549 and HEK293T in Dulbecco's modified Eagle's medium

(DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin. Small interfering RNA (siRNA) was transfected using Lipofectamine RNAiMAX (Life Technologies). Lentiviral transduction of shRNA or β-catenin was performed as described previously (Ma et al., 2012). Plasmids encoding wild-type or K314R mutant RelA and empty vector pcDNA3.1 were transfected using TransIT-LT1 Transfection Reagent (Mirus). Cells were pre-treated with or without 200 ng/ml WNT-3A before the addition of 1 ng/ml IL-1β. Total stimulation times before sample harvest are indicated for each experiment.

RNA isolation, cDNA synthesis and qRT-PCR

Total RNA was isolated using the NucleoSpin RNA II kit (Macherey-Nagel). cDNA was synthesized with the High Capacity cDNA Reverse Transcription Kit (Life Technologies). Quantitative real-time PCR (qRT-PCR) was performed using SYBR Green and Rotor-Gene 3000 (Qiagen), with GAPDH as the internal control.

Protein analysis and chromatin immunoprecipitation

Total proteins were isolated using RIPA lysis buffer supplemented with cOmplete Protease Inhibitor Cocktail (Roche). For acetylated RelA, 1 μM trichostatin A (TSA), 2 mM nicotinamide and 5 mM sodium butyrate were included. Nuclear and cytoplasmic extracts were prepared as described previously (Rothgiesser et al., 2010a). For immunoprecipitation, nuclear extracts and protein G Dynabeads (Life Technologies) pre-coupled with antibodies were incubated in immunoprecipitation buffer (25 mM HEPES pH 7.5, 150 mM NaCl, 2 mM MgCl$_2$, 0.5% NP-40 and Roche cOmplete) for 6 h at 4°C on a roller. After washing three times with immunoprecipitation buffer, proteins were eluted with Laemmli buffer and subjected to SDS-PAGE and western blotting. Interaction of recombinant proteins was assessed after incubation in immunoprecipitation buffer for 30 min at 30°C as described for endogenous proteins. Immunostaining of paraformaldehyde-fixed cells and chromatin immunoprecipitation were performed as described previously (Lehmann et al., 2015; Santoro et al., 2002).

In vitro acetylation assay

Recombinant RelA (1 mg) and CBP (500 ng) were incubated with or without β-catenin (1 mg) in HAT buffer (50 mM Tris-HCl pH 8, 100 mM NaCl, 10% glycerol, 1 mM PMSF, 1 mM DTT, 1 mM sodium butyrate and Protease Inhibitor Cocktail) in the presence or absence of 150 mM acetyl-CoA. After 1 h at 30°C, samples were subjected to SDS-PAGE and western blotting.

Statistical analysis

Data are expressed as the mean±s.d. and were analyzed by Student's two-tailed *t*-tests. $P<0.05$ were considered to be significant.

Supplementary material

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.168542/-/DC1

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