BCL-3 promotes a cancer stem cell phenotype by enhancing β-catenin signalling in colorectal tumour cells

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Summary statement

BCL-3 acts as a co-activator of β-catenin/TCF-mediated transcriptional activity driving a stem cell-like phenotype in colorectal cancer cells: implications for tumour cell plasticity and therapeutic resistance.

Abstract

To decrease bowel cancer incidence and improve survival, we need to understand the mechanisms that drive tumorigenesis. Recently BCL-3 (a key regulator of NF-κB signalling) has been recognised as an important oncogenic player in solid tumours. Although reported to be over-expressed in a subset of colorectal cancers (CRC), the role of BCL-3 expression in colorectal tumorigenesis remains poorly understood. Despite evidence in the literature that BCL-3 may interact with β-catenin it is perhaps surprising, given the importance of deregulated Wnt/β-catenin signalling in colorectal carcinogenesis, that the functional significance of this interactions is not known. Here we show for the first time that BCL-3 acts as a co-activator of β-catenin/TCF-mediated transcriptional activity in colorectal cancer cells and that this interaction is important for Wnt-regulated intestinal stem cell gene expression. We demonstrate that targeting BCL-3 expression (using RNA interference) reduced β-catenin/TCF-dependent transcription and the expression of intestinal stem cell genes *LGR5* and *ASCL2*. In contrast, the expression of canonical Wnt-targets C-Myc and Cyclin D1 remained unchanged. Furthermore, we show that BCL-3 increases the functional stem cell phenotype as shown by colorectal spheroid and tumoursphere formation in 3D culture conditions.

We propose that BCL-3 acts as a driver of the stem-cell phenotype in CRC cells potentially promoting tumour cell plasticity and therapeutic resistance. As recent reports highlight the limitations of directly targeting cancer stem cells (CSC), we believe that identifying and targeting drivers of stem cell plasticity have significant potential as new therapeutic targets.

Introduction

In 2014, there were around 15900 deaths attributed to bowel cancer in the UK, placing it second when ranked against all cancer mortalities (Cancer Research UK, 2014). To decrease bowel cancer incidence and improve survival, we need to develop new approaches to cancer treatment. To do this it is critical that we increase our understanding of the biology of the early stages of human colorectal tumorigenesis. Important recent studies in stem cell biology (Koo and Clevers, 2014) have begun to identify the mechanisms underpinning the drive towards or expansion of mutant stem cells that contribute to the earliest stages of tumour development in mice (Philpott and Winton, 2014). Importantly, environmental factors including the inflammation and activation of NF-κB signalling can promote the expansion of mutant cell populations, contributing both towards the earliest stages of tumorigenesis, but also to the maintenance of the tumour, and subsequently to therapeutic resistance and ultimately poor prognosis (Schwitalla et al., 2013, Grivennikov et al., 2010, Vlantis et al., 2011, Shaked et al., 2012).

Unsurprisingly, given its importance in gut development, maintenance and homeostasis (Clevers and Nusse, 2012), deregulation of Wnt signalling is an initiating factor in colorectal tumorigenesis; with *APC* mutation being the most frequent event in colorectal cancers (Segditsas and Tomlinson, 2006). During active signalling, the Wnt effector protein β-catenin translocates from the cytoplasm to the nucleus and binds TCF/LEF transcription factors situated at promoters of Wnt-responsive genes. β-catenin recruits other co-activators such as CBP (Li et al., 2007) and BCL-9 (Sustmann et al., 2008) to initiate transcription of genes involved in proliferation or stemness that are otherwise silent in the absence of Wnt ligands (Valenta et al., 2012). Co-activators that bind to the C-terminus of β-catenin are diverse in their methods of transcriptional activation and include chromatin-remodelling enzymes, histone acetyl-transferases and histone methyl-transferases (Valenta et al., 2012). CRCs frequently occur through inactivating mutations of the tumour suppressor APC (part of the 'destruction complex' that degrades β-catenin) or less-frequently via stabilizing mutations in β-catenin itself (Cancer Genome Atlas Network, 2012), consequently resulting in deregulated β-catenin signalling. Although there are many proteins that interact with β-catenin to influence its role in the cell (Valenta et al., 2012), it was of interest that a solitary panel in a figure of a paper by Kim *et al.* suggested that β-catenin may interact with the NF-κB co-regulator BCL-3 (either directly or indirectly), although the significance of the interaction was not described (Kim et al., 2005).

The B-Cell Chronic Lymphocytic Leukaemia/Lymphoma 3 (BCL-3) protein is highly expressed in a subset of CRCs where we have recently shown it inhibits apoptosis and promotes tumour growth (Urban et al., 2015). The *BCL3* gene was first discovered through cloning and sequencing of recurring t(14;19)(q32.3;q13.1) translocations identified in chronic lymphocytic leukaemia patients (McKeithan et al., 1990). It was predicted to encode a protein with a molecular weight of around 47kDa, with a proline-rich N-terminal domain, 7 central tandem-repeat cdc10 domains (ankyrin repeat domains) and a serine and proline-rich C-terminal domain (Ohno et al., 1990). BCL-3 is an atypical member of the inhibitor of kappa B (IκB) family of proteins and has been demonstrated to modulate transcription of NF-κB target genes via binding to homo-dimeric subunits of p50 or p52 through its ankyrin-repeat domains (Wulczyn et al., 1992, Bours et al., 1993). The p50/p52 subunits possess DNA-binding motifs, known as the Rel

homology domain, enabling them to occupy κB sites at promoters of NF-κB-responsive genes (Pereira and Oakley, 2008). This permits BCL-3 to activate (through its own transactivation domain or via recruiting alternative co-activators) or repress gene transcription (Dechend et al., 1999).

Under homeostatic conditions, BCL-3 plays important roles in the immune system and regulation of inflammation. Evidence of these functions were provided by Bcl-3 knockout mice which display defects in germinal centre development, a failure to generate IFN-γ-producing T-cells and an inability to produce antigen-specific antibodies in response to infection by certain bacterial species (Schwarz et al., 1997, Franzoso et al., 1997). Interestingly, Bcl-3$^{-/-}$ mice treated with DSS develop less severe colitis compared to wild type mice (O'Carroll et al., 2013); however, it has also been demonstrated that BCL-3 suppressed expression of pro-inflammatory cytokines in macrophages, dendritic cells and B cells in response to LPS-mediated TLR activation (Carmody et al., 2007), thereby demonstrating a complex role for BCL-3 in regulation of inflammation.

Although first characterised in hematopoietic cancers (Ohno et al., 1990), there is an emerging role for BCL-3 in solid tumours. It has been implicated in cancers arising in multiple tissue types including breast (Cogswell et al., 2000), prostate (Ahlqvist et al., 2013), cervical (Maldonado et al., 2010) and colorectal (Urban et al., 2015). BCL-3 bears numerous tumour-promoting capabilities such as increasing proliferation (Na et al., 1999), inflammation (Chang and Vancurova, 2014), inhibiting apoptosis (Kashatus et al., 2006) and promoting metastasis (Wakefield et al., 2013). Importantly, studies by Puvvada *et al.* and more recently by Saamarthy *et al.* report that around 30% of colorectal tumours present with nuclear BCL-3 (Puvvada et al., 2010, Saamarthy et al., 2015).

Considering the importance of Wnt/β-catenin and NF-κB crosstalk in cellular de-differentiation and tumour initiation in the intestine (Schwitalla et al., 2013), surprisingly there have been no studies exploring the role of NF-κB co-regulator BCL-3 in Wnt signalling in CRC. Here we report that BCL-3 is an important co-activator of β-catenin/TCF-mediated transcriptional activity in CRC cells. We show that BCL-3 regulates β-catenin-mediated transcription and expression of colorectal stem cell and cancer stem cell marker genes *LGR5* and *ASCL2*, functionally promoting a stem cell phenotype in colorectal cancer cells.

We propose that BCL-3 acts as a driver of the stem-cell phenotype in CRC cells, potentially promoting tumour cell plasticity and therapeutic resistance. As recent reports highlight the limitations of directly targeting cancer stem cells (CSC), we believe that identifying and targeting drivers of stem cell plasticity (Shimokawa et al., 2017) have significant potential as new therapeutic targets.

Results

β-catenin regulates BCL-3 expression in CRC cells

To initially demonstrate the importance of BCL-3 expression for patient outcome in CRC, we carried out survival analysis in relation to BCL-3 expression by using a publicly available colorectal cancer dataset (GSE24551) and Progene V2 (Goswami and Nakshatri, 2014), results are displayed in Figure 1A. Survival analysis in this dataset revealed high BCL-3 expression was linked to significantly reduced survival when adjusted for tumour stage. To examine the role of BCL-3 in Wnt/β-catenin signalling, we first screened a panel of human adenoma and carcinoma-derived cell lines for expression of BCL-3 and β-catenin (Figure 1B). Results show that both adenoma and carcinoma derived cell lines express β-catenin and BCL-3 protein, although there is an apparent inverse correlation between β-catenin and BCL-3 protein levels (those cells with lowest β-catenin generally having higher levels of BCL-3). To determine whether BCL-3 is regulated by β-catenin expression, LS174T cells with a doxycycline-inducible shRNA targeted towards β-catenin (a kind gift from Professor Hans Clevers, Utrecht) were used to suppress β-catenin expression. LS174T/R1 control cells were transfected with an otherwise identical plasmid, which expressed a non-targeting shRNA upon doxycycline addition. Protein levels of β-catenin and BCL-3 were analysed by western blot following 72 hours of doxycycline treatment. Results are displayed in Figure 1C. Doxycycline addition in the LS174T/sh-β-catenin cells resulted in efficient suppression of total and active β-catenin from 48 hours, with expression even further reduced by 72 hours. No reduction in β-catenin protein was detected in sh-β-catenin cells without doxycycline treatment. Interestingly, BCL-3 expression was strongly induced following β-catenin suppression at 48 and 72 hour timepoints following doxycycline addition suggesting that BCL-3 expression is repressed by Wnt/β-catenin signalling.

As off-target effects are possible when using siRNA or shRNA to target mRNAs (Jackson and Linsley, 2010), LS174T cells were selected and transfected with two independent siRNA sequences targeting β-catenin. One of these siRNAs (β-catenin siSTABLE) has enhanced stability within the cell. Cells were treated with control and β-catenin siRNA for 72 hours. Expression of BCL-3 was analysed by western blot (Figure 1D). Efficient β-catenin suppression was observed from 24 hours onwards with both β-catenin targeting siRNAs. BCL-3 upregulation was detected in response to β-catenin suppression with both sequences and at all timepoints analysed, in agreement with results in Figure 1C. Together, these results show that BCL-3 expression is increased following β-catenin suppression.

BCL-3 interacts with β-catenin and regulates β-catenin/TCF reporter activity in colorectal cancer cell lines

To investigate any potential interaction between BCL-3 and β-catenin in CRC cells, we selected the *APC* mutant SW1463 cell line for its relatively high endogenous expression of both BCL-3 and β-catenin. We performed BCL-3 Co-IPs on nuclear-enriched lysates and were able to identify an interaction between endogenous BCL-3 and β-catenin previously unreported in CRC cells (Figure 2A). CYLD was included as a positive control of BCL-3 binding. In addition, as it has previously been demonstrated that TNF-α can induce BCL-3 binding to p52 homodimers (Zhang et al., 2007), we treated

SW1463 cells with TNF-α for 6 hours to activate NF-κB signalling and carried out BCL-3 Co-IPs in the resulting lysates. In stimulated cells we detected an interaction between BCL-3 and p52 and again demonstrated the association of BCL-3 and β-catenin (Figure 2B). The interaction was further detected by β-catenin Co-IPs in control and TNF-α treated SW620 cells (figure 2C and D; TCF4 was included as a positive control for β-catenin interaction). These data suggest endogenous BCL-3 interacts with β-catenin in CRC cells.

We next investigated the effects of BCL-3 expression on β-catenin/TCF-mediated transcription. To do this we used siRNA to suppress *BCL3* expression in colorectal cell lines before transfecting cells with TOPFlash reporter plasmid to measure β-catenin/TCF-mediated transcriptional output. Interestingly, we discovered a significant decrease in TOPFlash activity in LS174T (colon-derived, mutant β-catenin), SW620 (lymph node-derived, mutant APC) and SW1463 (rectal-derived, mutant APC) cell lines (Figure 3A). These data indicate that BCL-3 can regulate β-catenin/TCF-mediated transcription in CRCs with common Wnt driver mutations. In addition, we examined the role of BCL-3 in RKO CRC cells, which are reported to harbour no activating Wnt pathway mutations and show no detectable TOPFlash activity under unstimulated conditions (da Costa et al., 1999). In agreement with preceding experiments, there was a significant decrease in Wnt3a-induced TOPFlash activity in RKO cells when BCL-3 expression was suppressed (Figures 3B and C). We next analysed the outcome of transient BCL-3 overexpression in colorectal cancer cells. Overexpression of BCL-3 in SW620 and LS174T cell lines harbouring activating Wnt pathway mutations did not show any regulation of TOPFlash reporter activity (data not shown). The same was true in unstimulated RKO cells. However, in RKO cells stimulated with Wnt3a, BCL-3 overexpression significantly enhanced β-catenin/TCF reporter activity (Figures 3D and E). These findings show that in a non-deregulated Wnt setting BCL-3 can modulate β-catenin/TCF-dependent transcription, suggesting Wnt3a-mediated transcriptional responses are enhanced by BCL-3.

BCL-3 regulates expression of stemness-associated Wnt targets

After establishing that BCL-3 regulates β-catenin/TCF-dependent transcription, we investigated the role of BCL-3 in Wnt target gene regulation. Wnt targets are heavily involved in the maintenance of stem cells in the colon (Clevers and Nusse, 2012), with *LGR5* and *ASCL2* providing key examples of genes that are expressed in intestinal stem cells, but not expressed in other cell types of the gut (Sato et al., 2009, van der Flier et al., 2009, Barker et al., 2007, Schuijers et al., 2015). With this in mind, we used qRT-PCR to analyse mRNA levels of some classical Wnt target genes—along with stemness-associated Wnt targets—on BCL-3 suppression in LS174T, SW620 and SW1463 cells. We found classical β-catenin/TCF targets such as c-MYC and Cyclin D1 were not significantly regulated by BCL-3 knockdown, whereas stem cell-specific Wnt target genes *LGR5* and *ASCL2* were significantly downregulated in all three cell lines (Figure 4A). Western analysis was used to measure LGR5 and ASCL2 protein expression at 48 and 72 hours post-BCL-3 siRNA transfection; we found clear downregulation of LGR5 and ASCL2 protein in all cell lines and at both time-points (Figure 4B). Interestingly, we saw no decrease in total or 'actively signalling' (de-phosphorylated) β-catenin levels. *LGR5* encodes a G-protein coupled receptor expressed in stem cells of the intestine and is also thought to identify cancer stem cells in CRC (Barker et al., 2007, Merlos-Suarez et al., 2011, Kemper et al.,

2012, Hirsch et al., 2013). To further confirm regulation of LGR5 by BCL-3—and to rule out any potential off-target effects of using a single siRNA sequence—we used a second BCL-3-targeting siRNA. LGR5 suppression by BCL-3 depletion was demonstrated using two independent siRNA sequences in SW620, LS174T and SW1463 cell lines (Figure 4C), indicating that BCL-3 regulates LGR5 expression in CRC cells with different mutational backgrounds. This result confirmed that BCL-3-mediated intestinal stem cell marker regulation was not the product of siRNA-mediated off-target effects. Using LS174T and SW620 cell lines stably-expressing a BCL-3 expression construct (termed LS174T[BCL-3] and SW620[BCL-3]) or empty vector (LS174T[pcDNA] and SW620[pcDNA]) we were able to show enhanced expression of BCL-3 moderately increased LGR5 expression (Supplementary Figure 1). Together, these data suggest that BCL-3 may be promoting β-catenin/TCF-dependent transcription at specific intestinal stemness-associated gene loci, which doesn't appear to be via increasing the pool of actively signalling β-catenin.

To investigate the role of NF-κB homodimers in BCL-3-mediated regulation of LGR5, we used RNAi to co-suppress BCL-3 and p50—and subsequently BCL-3 and p52—in LS174T cells (Figure 4D and E). BCL-3 was still able to regulate LGR5 expression in both p50- and p52-depleted cells. These results indicate that BCL-3-mediated regulation of LGR5 is independent of NF-κB p50/p52 homodimers.

BCL-3 does not mediate β-catenin activity through promoting nuclear translocation or altering levels of LEF1

Having shown nuclear interaction between BCL-3 and β-catenin, and given that BCL-3 has been shown to promote NF-κB nuclear translocation (Zhang et al., 1994), to further examine how BCL-3 regulates β-catenin/TCF activity and target gene expression we investigated whether BCL-3 enhances β-catenin nuclear localisation. We performed western analysis on LS174T and SW1463 nuclear/cytoplasmic-enriched lysates following BCL-3 suppression. BCL-3 knockdown did not affect β-catenin nuclear translocation in either cell line (Figure 5A). We also analysed expression levels of TCF4 and LEF1, as diminished nuclear levels of these transcription factors may have a profound effect on transcription of Wnt target genes. We found no consistent regulation of TCF4, but a small decrease in LEF1 expression (Figure 5A). *LEF1* is a Wnt target gene and encodes a transcription factor that mediates β-catenin signalling (Hovanes et al., 2001, Filali et al., 2002). To investigate the role of LEF1 in BCL-3-mediated LGR5 regulation we used RNAi to suppress LEF1 in SW1463 cells and analysed LGR5 expression using western blotting. We found LEF1 knockdown had no effect on LGR5 levels (Figure 5B). These findings signify that modulation of β-catenin/TCF-dependent transcription and regulation of stemness-associated Wnt targets by BCL-3 is not through promotion of β-catenin, TCF4 or LEF1 nuclear translocation. When taken alongside our data from Co-IP and TOPFlash experiments (Figures 2 and 3), these results support the idea that BCL-3 may be acting as a transcriptional co-activator of the β-catenin/TCF complex. To test this, we performed BCL-3 and TCF4 ChIP in SW1463 cells using primers designed to specific regions in the *LGR5* promoter. LGR5 was first demonstrated as a β-catenin/TCF4 target gene by Van der Flier *et al.* (Van der Flier et al., 2007); primers were designed to encompass TCF4 consensus sites (Hoverter and Waterman, 2008) in the *LGR5* promoter region. The *LGR5* TCF4 -950 and the *LGR5* TCF4 -925 primers span a TCF4 binding site around 950bp upstream of the LGR5

transcriptional start site (TSS). The *LGR5* +520 primer set span a region of 150bp from around 500bp in a distal region downstream of the *LGR5* TSS, a potential regulatory region for LGR5 transcription, as determined by using TCF4 ChIP-seq and H3K4Me1 methylation data tracks on the UCSC Genome Browser (http://genome.ucsc.edu/) (Kent et al., 2002) as a reference. The *GPRC5A* DS primer set is designed to a 150bp region located 30760bp downstream of the *GPRC5A* TSS. This primer set was used as a negative control as it was designed to a non-promoter/non-enhancer region of a non-relevant gene (Greenhough et al., 2018).

Immunoprecipitation was performed using antibodies to BCL-3, IgG (negative control) and positive controls, acetylated histone H3 and TCF4. Acetylated histone H3 is a marker of permissive, or 'active' chromatin from which genes can be transcribed (Workman and Kingston, 1998, Yan and Boyd, 2006). Results are displayed in Figure 5C. TCF4 was located at all 3 regions of the *LGR5* promoter analysed and was absent from the *GPRC5A* downstream region. BCL-3, however, was not detected at the -950bp or -925bp regions. Interestingly, BCL-3 could be detected at the +520bp region, suggesting an increase in β-catenin/TCF4 activity via binding to this region in colorectal cancer cells. The high levels of histone H3 acetylation at the +520 region are consistent with those seen at enhancer regions in other sites of the genome (Creyghton et al., 2010). Together, these results suggest that BCL-3 can be detected at the +520 region of the LGR5 promoter, consistent with its role as a transcriptional co-activator.

BCL-3 regulates colorectal spheroid and tumoursphere formation in 3D culture

Given that BCL-3 promotes LGR5 and ASCL2 expression, we investigated the functional consequence of stemness marker regulation using an adapted version of the organoid model system pioneered by Sato *et al.* (Sato et al., 2009) to grow CRC cell lines as 3D spheroids. It has been shown that single LGR5-positive cells can form organoids in Matrigel (Sato et al., 2009, Barker et al., 2010). As BCL-3 promotes LGR5 expression, we hypothesised that suppressing BCL-3 may inhibit the ability of single cells to progress and form spheroids. SW1463 cells were used as they form lumenal spheroids in 3D culture (Figure 6B), suggesting the presence of differentiated cell types in addition to cancer stem cells (Yeung et al., 2010, Ashley et al., 2013). Additionally, SW1463 cells express high levels of BCL-3 and undergo efficient BCL-3 suppression following siRNA transfection, increasing the likelihood of observing a strong phenotypic effect. Equal numbers of viable control and BCL-3 siRNA treated cells were re-suspended in Matrigel and seeded into 48 well plates. We then measured the number of spheroids formed after 10 days of culture. At the time of seeding, duplicate flasks were lysed and checked for efficacy of BCL-3 knockdown. We found suppression of BCL-3 and downregulation of LGR5 was achieved after 24 hours (Figure 6A) and strong suppression of BCL-3 was maintained following 84 hours of BCL-3 knockdown (Supplementary Figure 2), ensuring BCL-3 was suppressed during the critical early stages for spheroid initiation. Furthermore, we noted a significant reduction in the number of spheroids formed upon BCL-3 suppression relative to controls following 10 days of growth (Figures 6B-D). We repeated this experiment using SW620[pcDNA] and SW620[BCL-3] cells to investigate the outcome of BCL-3 overexpression on spheroid formation. SW620 cells were chosen for BCL-3 overexpression experiments in 3D conditions as they have low endogenous levels of BCL-3; we hypothesised that

overexpressing BCL-3 in a low endogenously-expressing cell line would have a greater effect than overexpressing BCL-3 in SW1463 cells which naturally express high endogenous levels of BCL-3 (enough endogenous protein to form functional interactions already). Of note, these cells were maintained on selection medium to ensure high constitutive overexpression of BCL-3 for the duration of the experiment (Supplementary Figure 1). We found BCL-3 overexpression significantly increased the number of spheroids formed following 10 days of culture (Figure 6E). These results suggest BCL-3 enhances the ability of cells to initiate full spheroid formation under these conditions, indicating that BCL-3 may promote stemness of CRC cells.

To further investigate the effect of BCL-3 on stemness of CRC cells we used a tumoursphere 3D culture system. Tumoursphere assays have been used previously to identify undifferentiated cancer stem cells in CRC (Ricci-Vitiani et al., 2007). We transfected SW1463 cells with control or BCL-3 siRNA for 24 hours before seeding into serum-free conditions in non-adherent 6 well plates and cultured for 6 days. The number of viable cells were counted at the end of this period to determine any effect BCL-3 might have on tumoursphere formation. Consistent with the findings above, we found BCL-3 suppression significantly reduced tumoursphere formation in serum-free conditions (Figure 6F). Furthermore, upon BCL-3 overexpression in the same system using SW620^{pcDNA} and SW620^{BCL-3} lines we reported a significant increase in the number of cells comprising the tumourspheres formed by BCL-3 overexpressing cells versus controls (Figure 6G). This suggests that by enhancing their ability to form tumourspheres in 3D culture conditions, BCL-3 enhances the stem-like potential of colorectal cancer cells.

Discussion

Given that BCL-3 is overexpressed in a subset of colorectal cancers and is linked with poor prognosis (Puvvada et al., 2010) and the Wnt pathway is deregulated in the vast majority of colorectal tumours (Segditsas and Tomlinson, 2006), the aim of this study was to investigate the role of NF-κB co-regulator BCL-3 in β-catenin/TCF-mediated signalling in colorectal cancer cells. Previously, survival of APC mutant stem cells had been shown to be promoted by NF-kB signalling (van der Heijden et al., 2016). However, initial Co-IP experiments demonstrated an interaction between BCL-3 and β-catenin, with further work illustrating BCL-3-mediated regulation of TOPFlash reporter activity and intestinal stem cell genes *LGR5* and *ASCL2*. Furthermore, this regulation was found to be independent of NF-κB homodimers, highlighting a novel link between NF-κB co-regulator BCL-3 and β-catenin/TCF-mediated signalling. Finally, colorectal spheroid and tumoursphere formation assays indicated that BCL-3 plays a functional role in enhancing stem-like potential of colorectal cancer cells.

Importantly, we show that BCL-3 suppression can downregulate β-catenin signalling in cells expressing mutant *APC* (the most common mutation in colorectal cancers) and mutant β-catenin itself (in addition to cells with non-mutated Wnt components), which suggests that targeting BCL-3 would reduce β-catenin signalling in colorectal tumours. The 'just right' model for Wnt signalling in colorectal cancer was suggested by Albuquerque *et al.* who observed the non-random distribution of mutational hits in *APC* in tumours from FAP patients. It was shown that some β-catenin binding activity (and resulting β-

catenin degradation) in one of the APC alleles was always retained, suggesting an optimal level of β-catenin/TCF-mediated transcription for tumour progression – as mutations that completely abolished the β-catenin binding ability of APC were not selected for (Albuquerque et al., 2002). By suppressing BCL-3, it is tempting to speculate that this might reduce the Wnt signalling level to below the 'just right' threshold in colorectal tumour cells, preventing deregulated transcription of select Wnt target genes.

In this report we demonstrate that BCL-3 regulated the expression of the β-catenin/TCF targets LGR5, ASCL2 and, to a lesser extent, LEF1. Conversely, expression of other canonical β-catenin/TCF targets analysed (Cyclin D1 and c-MYC) was not modulated by BCL-3. The BCL-3-mediated regulation of a specific subset of intestinal stem cell and Wnt target genes, and not of proliferation-inducing Wnt targets, is unusual given that suppression of BCL-3 expression inhibits total β-catenin activity as shown by TOPFlash reporter activity in colorectal cancer cells. However, transcriptional regulation of Wnt target genes from genomic DNA is more complex than the simple binding of TCF/LEF to DNA and availability of free β-catenin to activate transcription. The vast number of proteins that interact with β-catenin to regulate its transcriptional activity—with some co-regulators only regulating a subset of Wnt targets—support this (Valenta et al., 2012). Preferential regulation of stem cell specific targets is not unheard of and has been shown recently in colorectal tumours with *PTPRK-RSPO3* fusions. Tumours harbouring RSPO2 and RSPO3 fusions were found to occur in around 10% of colorectal tumours and result in upregulation of RSPO2 or RSPO3 expression. Additionally, they do not contain other Wnt-activating mutations (Seshagiri et al., 2012). Upon treatment of these tumours with anti-R-SPO3 antibody using patient derived xenograft models, expression of *LGR5* and *ASCL2* were highly downregulated, whereas *c-MYC*, *AXIN2* and *CCND1* (Cyclin D1) did not rank among the top 100 downregulated genes (Storm et al., 2016). Additionally, previous work has shown that Wnt co-activator BCL9/9L promotes activation of stemness-related target genes and not of proliferation-inducing Wnt targets (Moor et al., 2015). As yet, the mechanism behind the selectivity of *LGR5* and *ASCL2* regulation over other canonical Wnt targets remains undefined and warrants further investigation. It may be governed by other co-regulators present in the transcriptional complex, as has been previously demonstrated by Kim *et al.* who showed both activation and repression of *KAI1* occurred in the presence of BCL-3, with activation or repression dependent on the co-regulator Pontin or Reptin, respectively (Kim et al., 2005).

By preferentially diverting β-catenin/TCF-mediated transcription towards stemness genes *LGR5* and *ASCL2* over some of the more classical Wnt targets such as Cyclin D1 and c-MYC, it appears that BCL-3 may be driving CRC cells towards a stem-cell phenotype. Not only are *LGR5* and *ASCL2* robust stem cell markers, but numerous studies have shown them to be upregulated in CRCs, with many reporting they are markers for CRC stem cells (Merlos-Suarez et al., 2011, Kemper et al., 2012, Kobayashi et al., 2012, Hirsch et al., 2014, Jubb et al., 2006, Ziskin et al., 2013, Shimokawa et al., 2017). The cancer stem cell hypothesis states there are cells within tumours capable of self-renewal, in addition to producing other heterogeneous, differentiated cell types that constitute the tumour mass (Clarke et al., 2006). Cancer stem cells fuel tumour growth and are thought to be responsible for tumour re-constitution in cases of relapse if they are not eradicated by initial treatments (Merlos-Suarez et al.,

2011, Beck and Blanpain, 2013). Therefore, cancer stem cells must be eliminated by future therapeutics for CRC. In this study, enhancing levels of BCL-3 in CRC cells promoted colorectal spheroid and tumoursphere formation, indicating an increase in CRC stem-like activity (Ricci-Vitiani et al., 2007). Similarly, BCL-3 suppression impaired the ability of single cells to progress to fully-formed spheroids and inhibited tumoursphere formation. Through using colorectal spheroid and tumoursphere forming assays we present functional evidence that BCL-3 is promoting cancer stem cell activity. Intriguingly, our findings complement those of a recent study by Chen *et al.* proposing that BCL-3 plays a key role in maintaining naïve pluripotency in mouse embryonic stem cells (Chen et al., 2015). The findings of the Chen *et al.* study, albeit in a non-cancer background, concur with our conclusion that BCL-3 is important in promoting stem-like activity. Crucially, factors promoting plasticity of cancer stem cells will be important for future therapeutics, as reversion of differentiated progeny to cancer stem cells expressing LGR5 in colorectal cancer has been identified as a barrier to treatment (Shimokawa et al., 2017).

Recent work has provided insight into limitations on the efficacy of directly targeting LGR5^{+} colorectal cancer stem cells in primary tumours. Using genetic reporters and lineage tracing, Shimokawa *et al.* revealed that consecutive, specific ablation of LGR5^{+} cancer stem cells in xenotransplanted colorectal cancer organoids led to the reversion of differentiated KRT20^{+} cells. These cells filled the niche vacated by the LGR5^{+} cells and began to re-express LGR5, causing initial decreases in tumour volume but eventual tumour reconstitution following cessation of treatment (Shimokawa et al., 2017). Data from this study suggest BCL-3 may play a role in the de-differentiation and reconstitution of the tumour. Targeting BCL-3 may be an effective mechanism to prevent the reversion of LGR5^{+} cells in colorectal tumours. This could be a way of targeting cellular plasticity and a means of maintaining inhibition of primary tumour growth in already established primary tumours, without the associated tissue toxicity caused by sustained direct targeting of LGR5 (Tian et al., 2011). Furthermore, there is evidence that targeting cellular plasticity can be effective—a study targeting BMI-1 expressing cells showed that this reduced the number of cancer initiating cells *in vivo* (Kreso et al., 2014). In addition, BMI-1 cells have been shown to produce LGR5^{+} cells following epithelial damage (Tian et al., 2011). Targeting niche factors that promote stemness and plasticity such as BMI-1 and BCL-3, may be an effective way to target colorectal cancer stem cells in primary tumours and prevent reversion of differentiated cells to LGR5^{+} cancer stem cells.

In terms of therapeutics, BCL-3 could be an exciting novel target for colorectal cancer therapy. The *Bcl-3^{-/-}* mouse develops normally and has no obvious intestinal phenotype (Schwarz et al., 1997), alluding to potentially fewer toxic side effects than targeting the β-catenin/TCF interaction directly, which is crucial for normal gut homeostasis and crypt maintenance (Valenta et al., 2012, Clevers and Nusse, 2012). Our data show that BCL-3-mediated regulation of LGR5 and ASCL2 is especially important in colorectal cancer. Furthermore, we would hypothesise that modulation of these targets by BCL-3 could play a role in other physiological circumstances. Stresses such as inflammation may increase BCL-3 expression (Brasier et al., 2001, Tohyama et al., 2017) resulting in enhanced Wnt signalling; with the link between inflammation and Wnt signalling being previously established (Schwitalla et al., 2013).

Current studies are focusing on revealing any potential implications for normal intestinal physiology through inflammation-induced regulation of BCL-3 expression. Data from this study in RKO cells (Figure 3) with a wild-type Wnt pathway suggests targeting this protein may help to prevent or reduce aberrant Wnt signalling at early stages of tumorigenesis, in addition to later stage cancers. Furthermore, BCL-3 appears to still modulate β-catenin/TCF-mediated transcription downstream of mutated APC (SW1463, SW620) and β-catenin (LS174T); two of the most frequent mutations in CRC (2012). If the BCL-3/β-catenin interaction could be mapped and disrupted, this may be an effective therapeutic approach for targeting cancer stem cell plasticity in colorectal cancer and may add to the current arsenal of Wnt-related inhibitors currently under development (Katoh, 2017).

In conclusion, we have shown for the first time that BCL-3 potentiates β-catenin/TCF-mediated signalling in CRC and selectively regulates transcription of intestinal stem cell genes and Wnt targets *LGR5* and *ASCL2*, promoting a cancer stem cell phenotype. Our data suggest BCL-3 may represent an exciting new avenue for targeting plasticity of cancer stem cells in CRC, particularly as it enhances β-catenin activity downstream of frequently mutated APC and β-catenin.

Materials and Methods

Cell lines

All cell lines used in this study were obtained from American Type Culture Collection (ATCC; Rockville, MD, USA). LS174T cells were established from a Duke's type B colonic adenocarcinoma; SW620 cells were derived from a lymph node metastasis of the primary colorectal tumour (Duke's type C colorectal adenocarcinoma); SW1463 cells were established from a Duke's type C rectal adenocarcinoma; RKO cells were established from a colorectal carcinoma. All cell lines routinely tested for mycoplasma contamination using MycoAlert PLUS mycoplasma detection kit (Lonza, MD, USA).

Cell culture

All cell lines were cultured in 10% FBS DMEM - Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Life Technologies, Paisley, UK) with added 10% foetal bovine serum (FBS) (GE Healthcare, UK), 2mM glutamine (Sigma-Aldrich, Dorset, UK), 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen, Life Technologies, Paisley, UK). LS174T$^{pcDNA/BCL-3}$ and SW620$^{pcDNA/BCL-3}$ cell lines were cultured in 10% DMEM with added 400µg/ml G418 (Sigma-Aldrich). For stock purposes cells were maintained in T25 flasks (Corning, NY, USA) and incubated at 37°C in dry incubators maintained at 5% CO_2. Cells were medium changed every 3-4 days.

SDS-PAGE and western analysis

Cell lysates were prepared and subjected to western analysis as described previously (Williams et al., 1993) using the following antibodies: α-Tubulin (T9026; Sigma-Aldrich; 1:10000), ASCL2 (4418; EMD Millipore, Watford, UK; 1:500), β-catenin (9587; Cell Signalling Technology, MA, USA; 1:5000), β-catenin (610153; BD Biosciences, CA, USA; 1:10000), de-phosphorylated (actively signalling) β-catenin

(05-665; EMD Millipore; 1:1000), BCL-3 (ab49470; Abcam, Cambridge, UK; 1:1000), BCL-3 (23959; Proteintech, Manchester, UK; 1:2000), c-MYC (sc-40; Santa Cruz Biotechnology, TX, USA; 1:500), Cyclin D1 (2978; Cell Signalling Technology; 1:1000), Lamin A/C (4200236; Sigma; 1:5000), LEF1 (2230; Cell Signalling Technology; 1:1000), LGR5 (ab75850; Abcam; 1:1000), NF-κB1 (p50; sc-8414; Santa Cruz Biotechnology; 1:1000), NF-κB2 (p52;05-361; EMD Millipore; 1:1000) and TCF4 (2569; Cell Signalling Technology; 1:1000).

Complex Immunoprecipitation (Co-IP)

BCL-3 CoIPs were performed in SW1463 nuclear-enriched lysates as previously described (Petherick et al., 2013). Briefly, 500µg of rabbit pan-IgG (12-370; EMD Millipore) pre-cleared nuclear-enriched protein lysates were cleared with 6µg of BCL-3 antibody (23959; Proteintech) conjugated to Dynabeads Protein A beads (Invitrogen) before undergoing western blot analysis. Beads were retrieved using a Dyna-Mag 2 magnet (Invitrogen). Immunoprecipitates were analysed by western blot for BCL-3 (ab49470; Abcam) and β-catenin (610153; BD Biosciences). For TNF-α treated CoIPs, 100ng/ml TNF-α (Source BioScience, Nottingham, UK) was added to cells for 6 hours prior to lysis. Immunoprecipitates were additionally analysed for p52 (05-361; EMD Millipore)

Gene knockdown via RNA interference (RNAi)

Cell lines were reverse transfected in Opti-MEM (Gibco) using RNAiMax (Invitrogen) with 50nM of siRNA, unless otherwise stated. Individual sequences and relevant non-targeting controls were used for silencing *BCL3* whereas a smart pool and non-targeting controls were used to silence *LEF1, NFKB1 and NFKB2*. All siRNA sequences were produced by Dharmacon (GE Lifesciences).

Quantitative reverse transcriptase-PCR (qRT-PCR)

Tri-Reagent (Sigma-Aldrich) was added to cells and RNAeasy mini kit (Qiagen, Limberg, Netherlands) was used according to manufacturer's instructions to clean up RNA before synthesis of cDNA and qRT-PCR were performed as previously described (Petherick et al., 2013) using the following primers: *BCL3* (QT00040040), *CTNNB1* (β-catenin) (QT01331274), *CMYC* (QT00035406), *CCND1* (Cyclin D1) (QT00495285), *LEF1* (QT00021133), *LGR5* (QT00027720). Gene expression was normalised to housekeeping gene *TBP* (QT00000721). *ASCL2* primer sequences (Sigma) were obtained from Jubb *et al.* (Jubb et al., 2006) (forward 5' – GGCACTGCTGCTCTGCTA – 3', reverse 5' – GTTCACGCTCCCTTGAAGA – 3').

Generation of stable BCL-3 expressing cell lines

Cells were transfected in Opti-MEM (Gibco) using Lipofectamine 2000 (Invitrogen) with pcDNA3-BCL-3 WT vector (Keutgens et al., 2010, Viatour et al., 2004) (kindly provided by Alain Chariot, University of Liège, Belgium; re-cloned by Tracey Collard, University of Bristol, UK) or empty pcDNA3 vector as a control. Resistant clones were selected and pooled in media supplemented with 500µg/ml neomycin.

TCF reporter assay (TOPFlash reporter assay)

BCL-3 expression was suppressed via RNAi and TOPFlash assay was performed as previously described (Petherick et al., 2013) using Promega Dual-Luciferase Reporter Assay System (Promega, WI, USA) according to manufacturer's instructions. Luminescence was measured at 560nm using a Modulus luminometer (Turner Biosciences, CA, USA). Co-transfection of FOPFlash reporter with mutated TCF consensus sites was used alongside TOPFlash to monitor non-specific output. In RKO cells 100ng/ml recombinant human WNT3a protein (R&D Systems, Abingdon, UK) was added to cells 48 hours post-siRNA transfection. For BCL-3 transient overexpression, cells were transfected with pcDNA3 control and pcDNA3-BCL-3 WT plasmids prior to Wnt3a treatment.

Spheroid forming assay

For BCL-3 knockdown experiments equal numbers of single SW1463 cells transfected with BCL-3 siRNA or negative control siRNA were re-suspended in Matrigel (Becton Dickinson, Oxford, UK) and seeded into 48 well plates (Corning), 24 hours post-transfection. Matrigel was submerged in spheroid medium consisting of Advanced DMEM:F12 (Gibco), 0.1% BSA (Sigma-Aldrich), 2mM glutamine (Sigma-Aldrich), 10mM HEPES (Sigma-Aldrich), 100 units/ml penicillin, 100 units/ml streptomycin, 1% N2 (Gibco), 2% B27 (Gibco) and 0.2% N-acetyl-cysteine (Gibco). Spheroids were cultured for 10 days as previously described (Sato et al., 2011). Wells were imaged using a DMI6000 widefield microscope (Leica Microsystems, Wetzlar, Germany) and Leica LAS-X acquisition software (Leica Microsystems). Images were analysed using Matlab R2015a software (Mathworks, MA, USA). Subjective gating was set to exclude any cells/debris less than 3000µm^2 in cross-sectional area. For BCL-3 overexpression experiments equal numbers of single SW620 cells stably-transfected with pcDNA-BCL-3 WT vector or empty pcDNA vector as a negative control were seeded into Matrigel and were cultured and analysed as described above.

Tumoursphere forming assay

For BCL-3 knockdown experiments SW1463 cells transfected with BCL-3 siRNA or negative control siRNA were counted using trypan blue (Invitrogen) and a Countess automated cell counter (Invitrogen) to exclude dead cells. 2x10^4 viable cells were re-suspended in tumoursphere medium consisting of Dulbecco's Modified Eagle Medium:F12 medium (DMEM:F12) (Gibco) supplemented with 20ng/ml EGF (Sigma-Aldrich), 10ng/ml FGF (R&D Systems), 2% B27 (Gibco), 400µg/ml G418, 2mM glutamine, 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen). Cells were seeded into non-adherent 6 well plates (Greiner Bio-One, Stonehouse, UK) and tumourspheres were cultured as described previously (Ricci-Vitiani et al., 2007) for 6 days before contents of wells were centrifuged for 3 minutes at 3000rpm. Tumourspheres were re-suspended in 500µL of StemPro Accutase cell dissociation reagent (Gibco) and manually dissociated via pipetting. Cells were incubated for 30 minutes to achieve single cell suspension and viable cells were counted using a Countess cell counter. For BCL-3 overexpression experiments SW620 cells stably-transfected with pcDNA-BCL-3 WT vector or empty pcDNA vector as a negative control were seeded, cultured and analysed as above.

Statistical analysis

Statistical analysis was performed using one sample t-test or Student's t-test as stated. Significance was expressed as *P<0.05, **P<0.01, ***P<0.001. Results are expressed as mean values with SEM.

Data availability

The publicly available dataset used in this study is available from NCBI Gene Expression Omnibus (GEO); GSE24551 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24551).

Author Contributions

Conception and design [ACW, DL, AS, TJC, CP, AG, RWC], execution [ACW, DL, AS, TJC, AG, ACC], analysis and interpretation of data [ACW, DL, AS, TJC, CP, AG, ACC] drafting the article [DL, AG, ACW] critical revision for important intellectual content [All].

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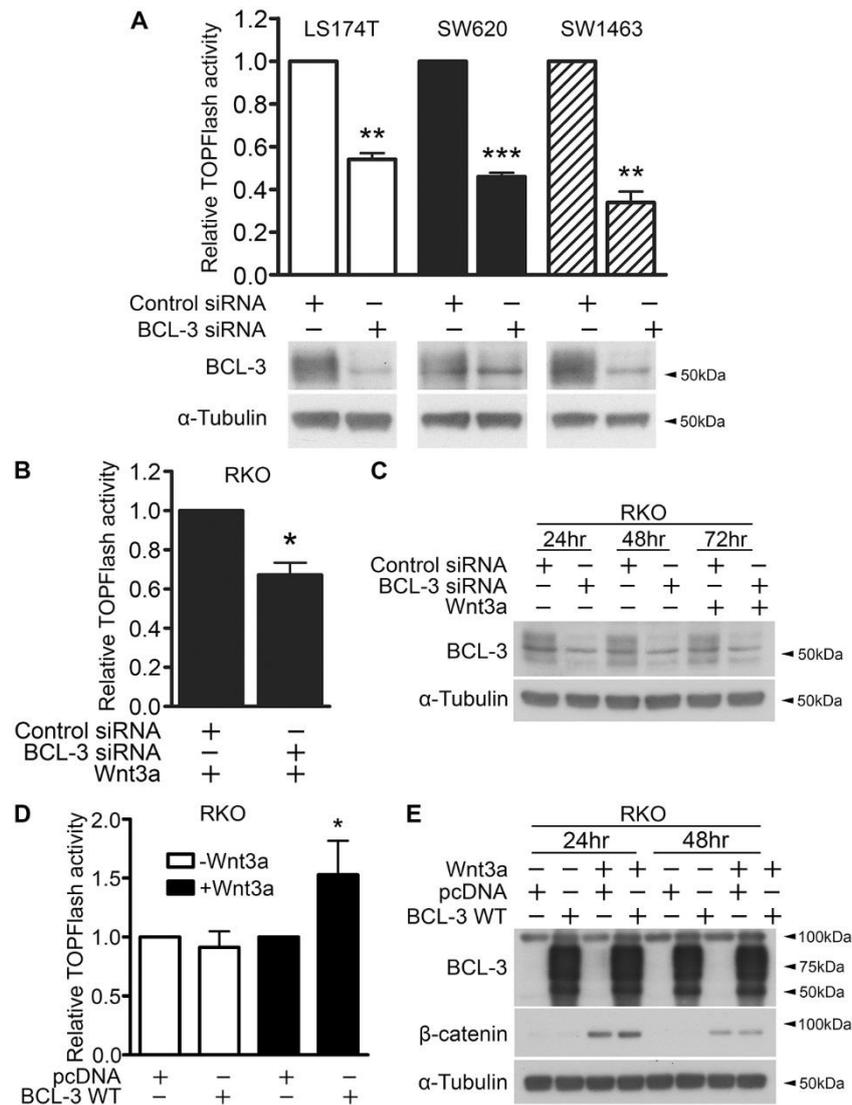
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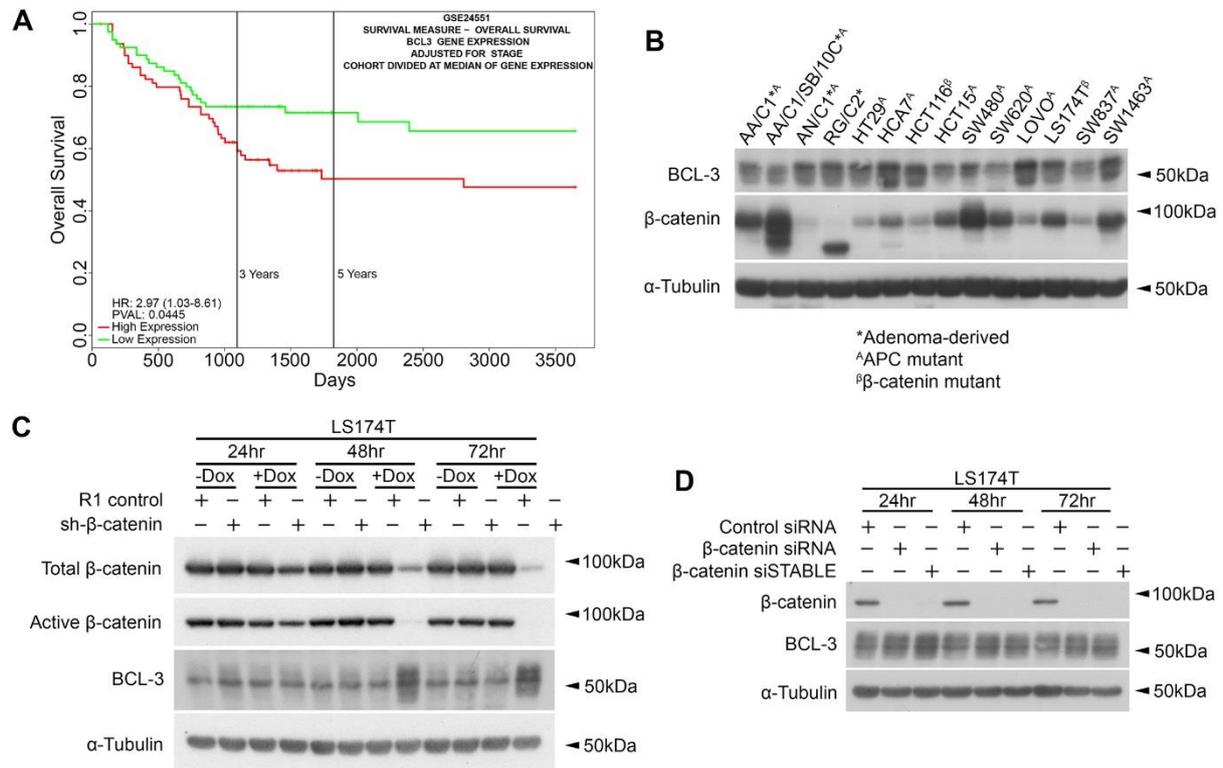
Figure 1. β-catenin regulates BCL-3 expression in colorectal cancer cells

(**A**) Survival analysis in relation to BCL-3 expression generated using publicly available colorectal cancer dataset (GSE24551) and Progene V2 (Goswami and Nakshatri, 2014). (**B**) Western blot analysis of adenoma- and carcinoma-derived colorectal cell lines showing expression of BCL-3 and β-catenin. α-Tubulin serves as a loading control. (**C**) Western analysis of total and active β-catenin and BCL-3 expression in LS174T cells with dox-inducible expression of β-catenin shRNA following 24, 48 and 72 hours of dox treatment (1μg/mL). LS174T/R1 cells possess a dox-responsive promoter upstream of a scrambled shRNA sequence and express a non-targeted shRNA upon treatment with dox. α-Tubulin serves as loading control. (**D**) Western analysis of β-catenin and BCL-3 expression in LS174T cells at 24, 48 and 72 hours post β-catenin siRNA transfection (25nM). β-catenin siSTABLE is a β-catenin targeted siRNA with enhanced stability. α-Tubulin serves as loading control. **Dox**, doxycycline.
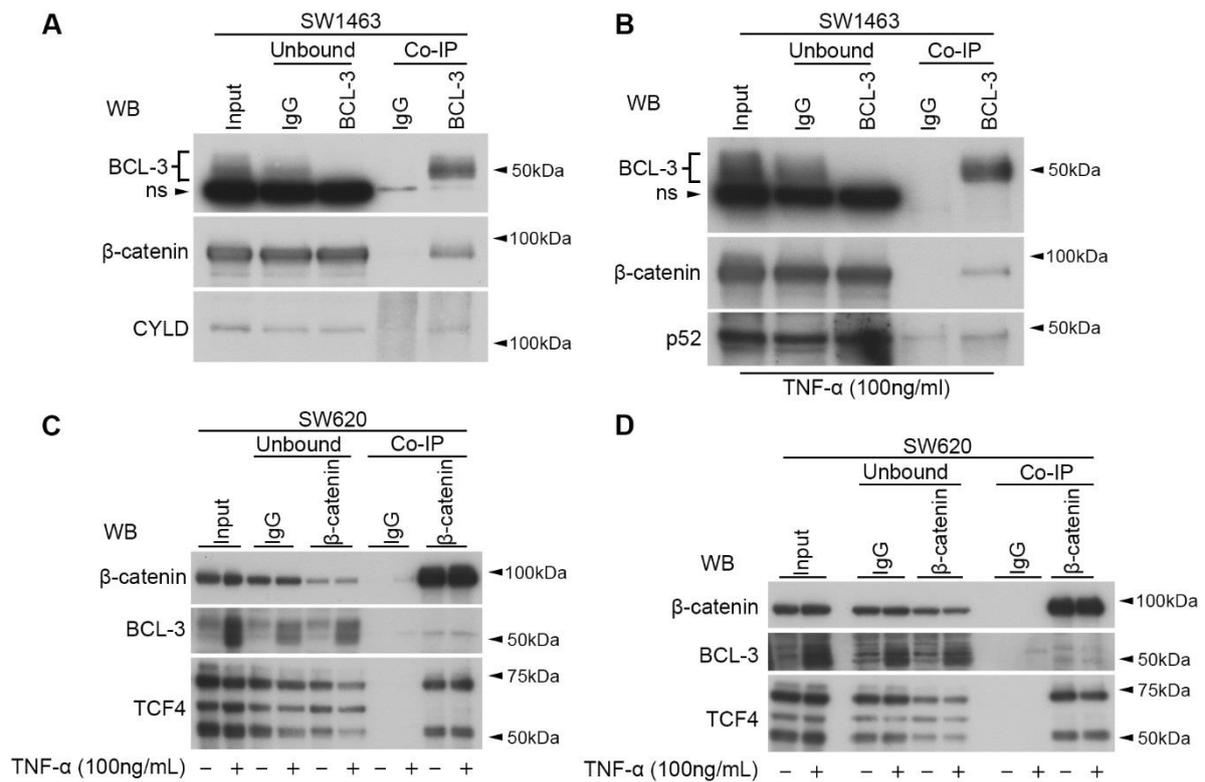
Figure 2. BCL-3 interacts with β-catenin in colorectal cancer cell lines

(**A**) and (**B**) BCL-3 Complex-immunoprecipitation (Co-IP) performed in SW1463 nuclear-enriched lysates. Unbound (Immuno-depleted lysates) shows depletion of proteins after IP. Immunoprecipitates were analysed by western blot for BCL-3 and β-catenin. IgG serves as negative control. (**A**) CYLD serves as positive control for BCL-3 binding (**B**) Cells were treated with 100ng/ml TNF-α for 6 hours prior to lysis. Immunoprecipitates were additionally analysed for p52. Note the presence of non-specific band in BCL-3 western analysis with use of Abcam BCL-3 antibody. (**C**) and (**D**) Nuclear β-catenin Co-IPs in SW620 cells. (**C**) and (**D**) represent experimental replicates. Western analysis of non-treated and 6 hour TNF-α treated cells following β-catenin Co-IP. TCF4 serves as a positive control for β-catenin binding. Mouse pan-IgG serves as negative control. **Unbound**, Immuno-depleted lysates following Co-IP; **WB,** western blot antibody; **ns**, non-specific band.

Figure 3. BCL-3 regulates β-catenin/TCF reporter activity

(**A**) β-catenin/TCF (TOPFlash) luciferase reporter assay. TOPFlash reporter activity measured 72 hours post-siRNA transfection in LS174T, SW620 and SW1463 carcinoma cells. Results are expressed as TOPFlash/Renilla. N=3, ± SEM **P<0.01, ***P<0.001 Western analysis shows expression of BCL-3. α-Tubulin serves as a loading control. (**B**) TOPFlash reporter activity measured 72 hours post-siRNA transfection in RKO cells. 100ng/ml Wnt3a was added to cells at 48 hours post-siRNA transfection. Results are expressed as TOPFlash/FOPFlash. One sample t-test. N=3, ± SEM *P<0.05. (**C**) Western analysis of BCL-3 expression over duration of TOPFlash assay performed in **B**. α-Tubulin serves as a loading control. (**D**) TOPFlash reporter activity measured 48 hours following BCL-3 overexpression and Wnt3a (100ng/ml) treatment. Results are expressed as TOPFlash/FOPFlash. One sample t-test. N=4, ± SD *P<0.05. (**E**) Western analysis of BCL-3 and β-catenin expression over duration of TOPFlash assay performed in (**D**). α-Tubulin serves as a loading control.
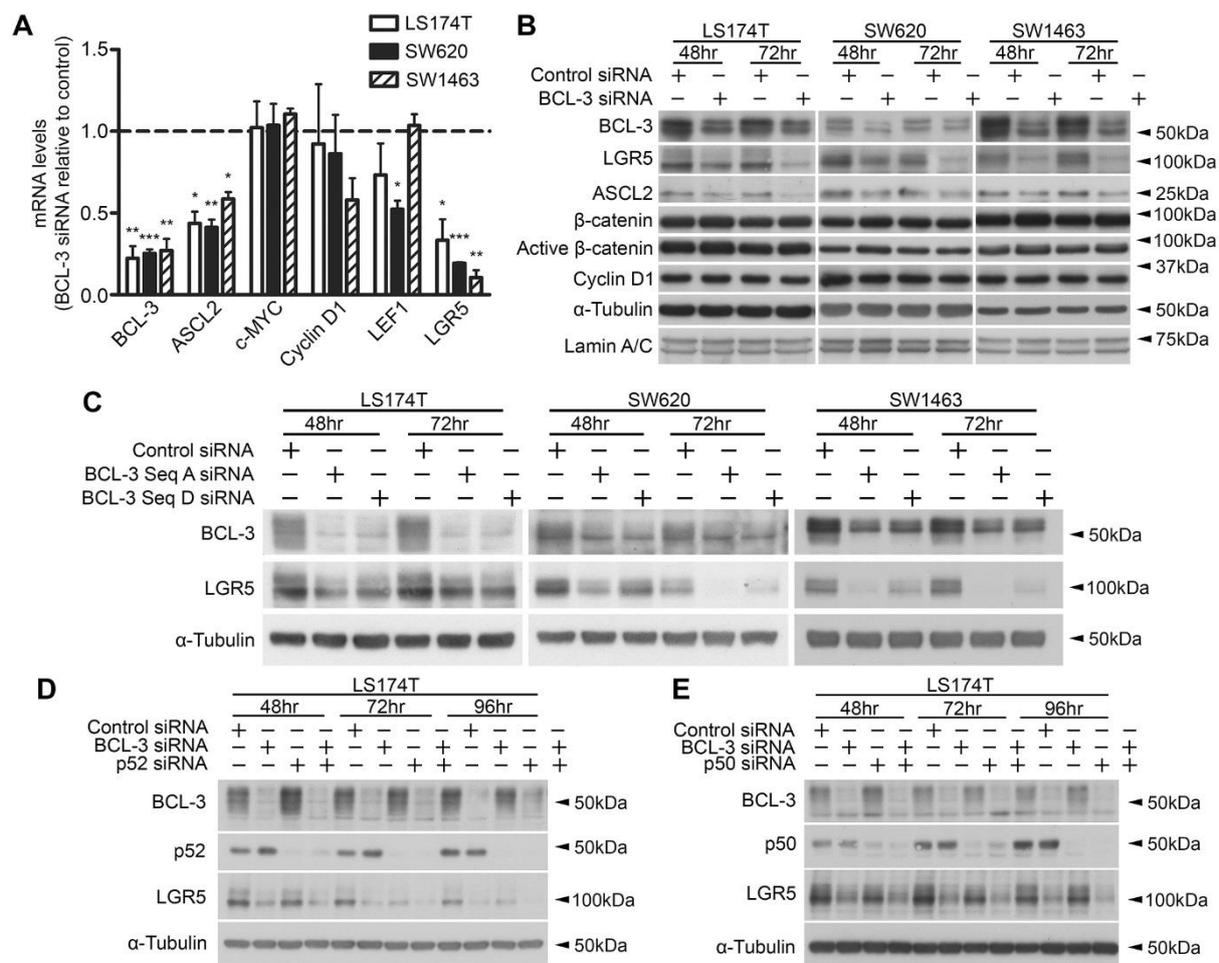
Figure 4. BCL-3 regulates expression of stemness-associated Wnt targets

(**A**) Quantitative reverse transcriptase-PCR (qRT-PCR) mRNA analysis of Wnt target gene expression in LS174T, SW620 and SW1463 cells following BCL-3 suppression. Data shows mRNA expression of genes normalised to housekeeping genes at 72 hours post-siRNA transfection, BCL-3 knockdown cells relative to controls. One sample t-test. $N=3$, ± SEM *$P<0.05$, **$P<0.01$, ***$P<0.001$. (**B**) Western analysis of LGR5 and ASCL2 expression in LS174T, SW620 and SW1463 cells following BCL-3 suppression. Expression of total β-catenin, de-phosphorylated (active) β-catenin and Cyclin D1 also shown. α-Tubulin serves as a loading control. ASCL2 expression is shown in nuclear-enriched lysates, with Lamin A/C serving as a loading control. (**C**) LGR5 expression in LS174T, SW620 and SW1463 cells following BCL-3 suppression with two independent siRNA sequences. LGR5 and BCL-3 expression analysed by western blot. α-Tubulin serves as a loading control. (**D**) Western analysis of BCL-3, p52 and LGR5 expression in LS174T cells at 48, 72 and 96 hours following siRNA transfection of indicated siRNAs (50nM). α-Tubulin serves as loading control. (E) Western analysis of LS174T cells for expression of BCL-3, p52 and LGR5 at 48, 72 and 96 hours following siRNA transfection of indicated siRNAs (50nM). α-Tubulin serves as loading control.
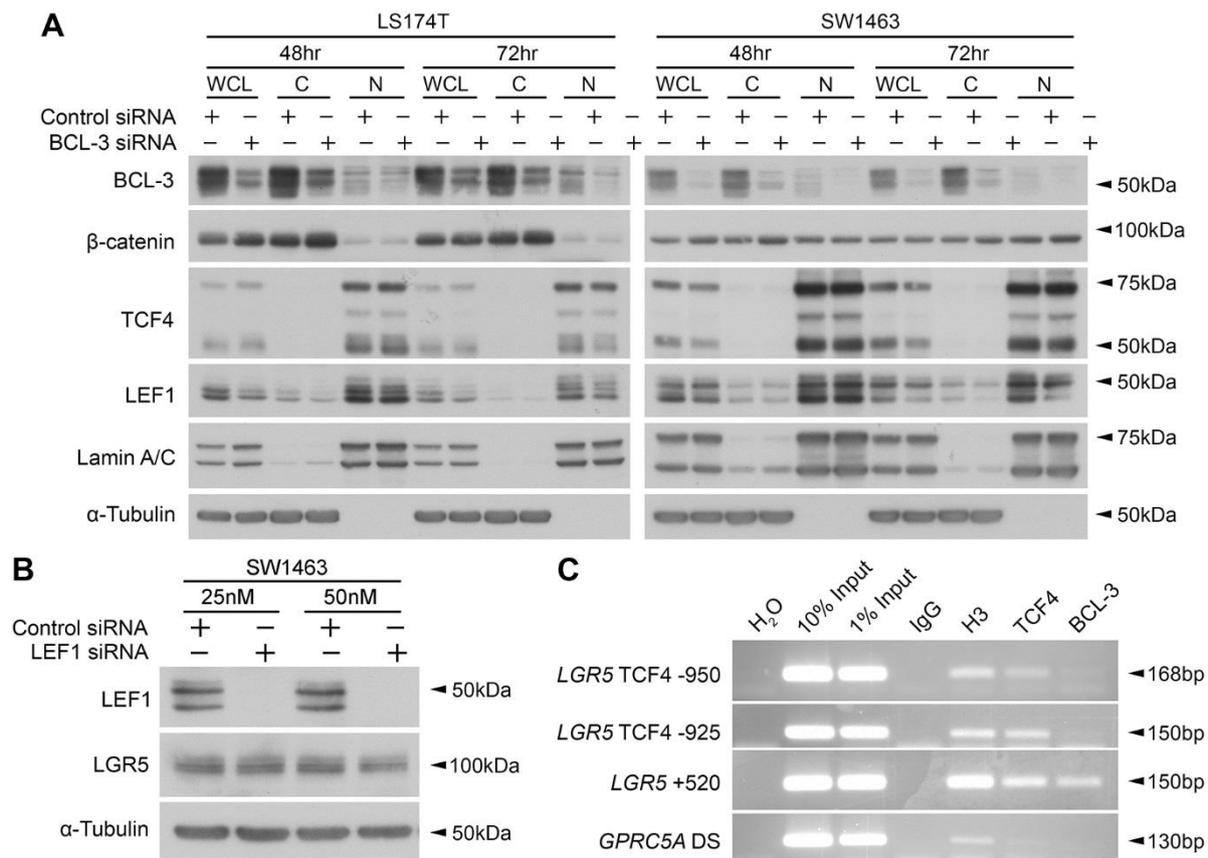
Figure 5. BCL-3 does not mediate β-catenin activity through promoting nuclear translocation or altering levels of LEF1

(**A**) Nuclear/cytoplasmic enrichments of LS174T and SW1463 cells following BCL-3 suppression showing localisation and expression of Wnt transcriptional regulators β-catenin, TCF4 and LEF1. Western analysis confirms BCL-3 suppression. α-Tubulin and Lamin A/C serve as loading controls and confirm cytoplasmic/nuclear enrichment. **WCL**, whole cell lysate; **C**, cytoplasm-enriched lysate; **N**, nuclear-enriched lysate. (**B**) LGR5 expression following LEF1 suppression with 25nM and 50nM siRNA in SW1463 cells. Data shown 48 hours post-siRNA transfection. α-Tubulin serves as a loading control. (**C**) ChIP analysis of 3 sites in the *LGR5* promoter in SW1463 cells for presence of TCF4 and BCL-3. H₂O serves as a negative control for PCR. IgG serves as negative control for immunoprecipitation (IP). Acetylated histone H3 serves as a positive control for IP. *GPRC5A* DS primers were designed to a downstream region in the *GPRC5A* gene with no reported transcription factor binding and serves as a negative primer control.
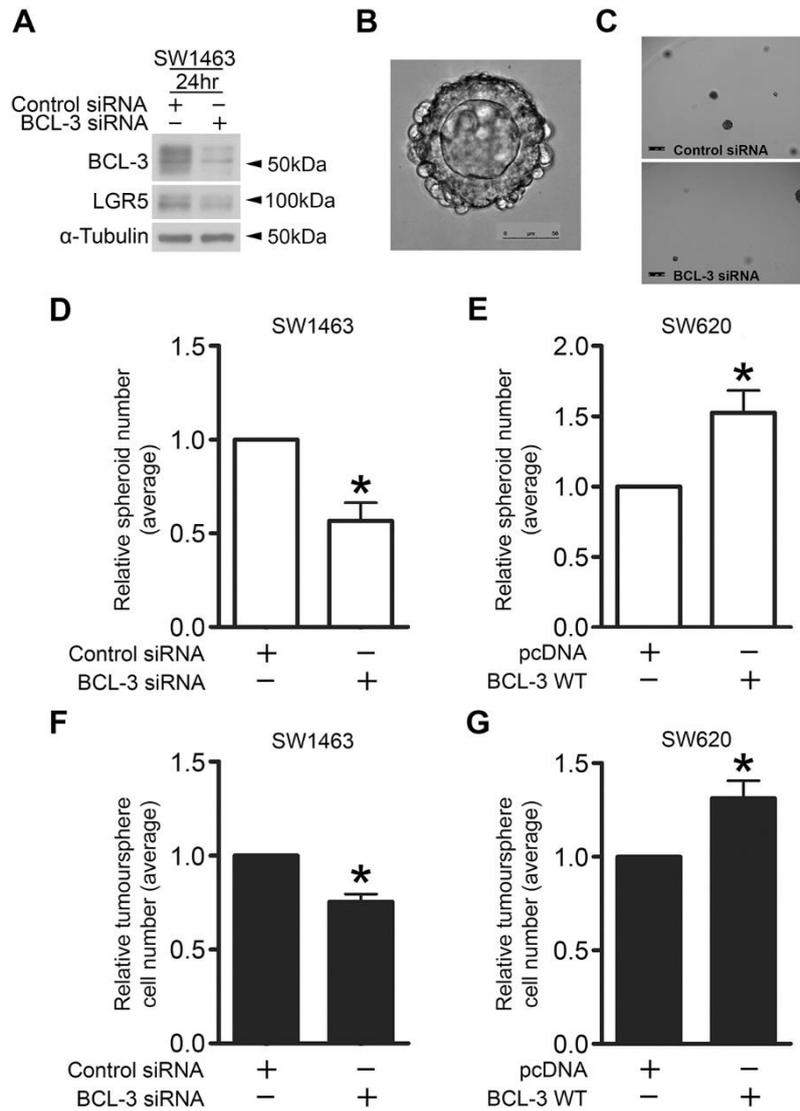
Figure 6. BCL-3 regulates colorectal spheroid and tumoursphere formation

(**A**) Western analysis confirming BCL-3 suppression and LGR5 downregulation in SW1463 cells seeded into Matrigel. α-Tubulin serves as a loading control. (**B**) Widefield microscopy image of a colorectal spheroid grown from a single SW1463 cell following 10 days of culture. 20x objective. Scale bar = 50μm. (**C**) Widefield microscopy images of wells containing BCL-3 knockdown and control SW1463 spheroids. 5x objective. Scale bars = 250μm. (**D**) and (**E**) Spheroid forming assay. (**D**) SW1463 cells transfected with BCL-3 siRNA or negative control siRNA were re-suspended in Matrigel and seeded into 48 well plates, 24 hours post-transfection. Spheroids were cultured for 10 days as previously described (Sato et al., 2011). Wells were imaged and analysed using Matlab R2015a software, with subjective gating applied to exclude any cells/debris less than 3000μm^2 in cross-sectional area. (Control siRNA 9.37 ± 4.51; BCL-3 siRNA 5.78 ± 4.02). One sample t-test. *N*=3, ± SEM *P<0.05. (**E**) SW620 cells stably-transfected with pcDNA-BCL-3 WT vector (SW620[BCL-3]) or empty pcDNA vector (SW620[pcDNA]) as a negative control were seeded into Matrigel, cultured and analysed as in (**D**). (pcDNA 4.15 ± 2.48; BCL-

3 WT 5.88 ± 2.40). One sample t-test. *N*=4, ± SEM **P*<0.05. (**F**) and (**G**) Tumoursphere forming assay. (**F**) SW1463 cells transfected with BCL-3 siRNA or negative control siRNA were re-suspended in tumoursphere medium and cultured as described previously (Ricci-Vitiani et al., 2007). Tumourspheres were dissociated and cells counted following 6 days of culture. (Control siRNA 8.93 ± 3.95×10^4; BCL-3 siRNA 6.61 ± 2.63×10^4). One sample t-test. *N*=3, ± SEM **P*<0.05. (**G**) SW620 cells stably-transfected with pcDNA-BCL-3 WT vector or empty pcDNA vector as a negative control were seeded, cultured and analysed as in (**F**). (pcDNA 3.83 ± 2.38×10^4; BCL-3 WT 4.81 ± 2.48×10^4). One sample t-test. *N*=4, ± SEM **P*<0.05.
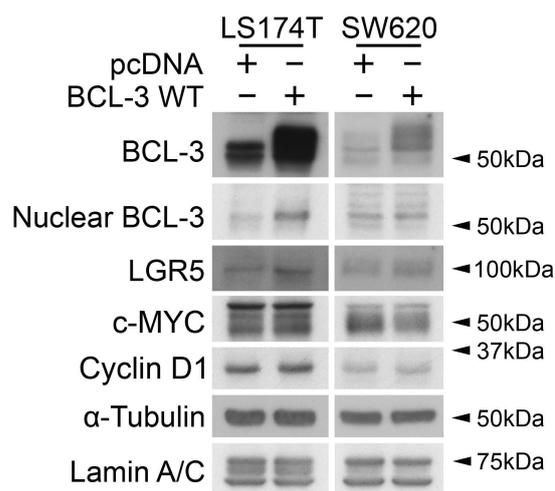
Figure S1. BCL-3 overexpression enhances LGR5 expression

LGR5 expression in LS174T and SW620 cells stably overexpressing BCL-3. LGR5, BCL-3, Cyclin D1 and c-MYC expression was analysed by western blot. α-Tubulin serves as a loading control. Nuclear BCL-3 expression was analysed in nuclear-enriched lysates with Lamin A/C serving as a loading control.
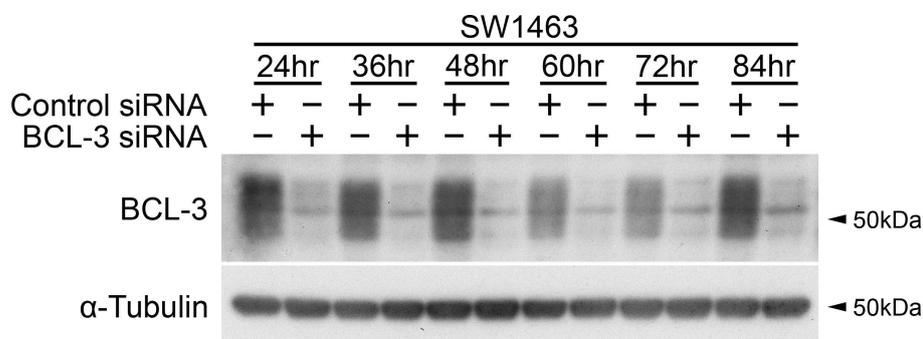

Figure S2. BCL-3 suppression is maintained at early stages of spheroid initiation

BCL-3 expression following 84 hours of BCL-3 knockdown in SW1463 cells grown as spheroids. α-Tubulin serves as loading control.