Cell cycle control of Wnt/ β -catenin signalling by conductin/axin2 through CDC20

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Wnt/ β -catenin signalling regulates cell proliferation by modulating the cell cycle and is negatively regulated by conductin/axin2/axil. We show that conductin levels peak at G2/M followed by a rapid decline during return to G1. In line with this, Wnt/ β -catenin target genes are low at G2/M and high at G1/S, and β -catenin phosphorylation oscillates during the cell cycle in a conductin-dependent manner. Conductin is degraded by the anaphase-promoting complex/cyclosome cofactor CDC20. Knockdown of CDC20 blocks Wnt signalling through conductin. CDC20-resistant conductin inhibits Wnt signalling and attenuates colony formation of colorectal cancer cells. We propose that CDC20-mediated degradation of conductin regulates Wnt/ β -catenin signalling for maximal activity during G1/S.

Keywords: axin 2; β -catenin; cell cycle; CDC20; Wnt signalling
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INTRODUCTION

Wnt/ β -catenin signalling functions in embryonic development, carcinogenesis and stem cell maintenance [1]. Wnt signalling increases the stability of β -catenin by inhibiting the ' β -catenin destruction complex'. In this complex, which includes the tumour suppressor adenomatous polyposis coli (APC) and scaffold proteins axin and conductin, β -catenin is sequentially phosphorylated by CK1 and GSK3 β at amino-terminal residues S45 and S33/37/T41, respectively, and degraded via the E3 ubiquitin ligase β -TrCP [2]. Activation of the pathway by binding of Wnts to Frizzled/LRP transmembrane receptors or by mutations in components of the complex (as seen in tumours) interferes with β -catenin degradation. Stabilized β -catenin enters the nucleus and through T-cell factor/lymphoid enhancer factor (TCF/LEF) cofactors directs transcription of target genes such as c-Myc and cyclin D1, which are important regulators of G1 to S cell cycle transition [2,3]. Indeed, inhibition of β -catenin-mediated transcription by a dominant-negative form of TCF4 arrests cells in G1 [4]. Furthermore, β -catenin levels increase during G1/S transition in non-transformed epithelial cell lines and during G1 to G2/M in a

variety of cell lines [5,6]. Phosphorylation of LRP6 by Cyclin Y and its related cyclin-dependent kinase (PFTK) is stimulated during mitosis, suggesting that the Wnt/ β -catenin signalling varies at different phases of the cell cycle [7]. We have previously shown that conductin (also known as axin2/axil) is a negative regulator, as well as a target gene, of Wnt signalling, thus acting in a negative feedback loop [8–10]. Conductin and other components of the Wnt/ β -catenin pathway localize and function at centrosomes and the mitotic apparatus, suggesting that the Wnt/ β -catenin pathway has evolved hubs for regulating different cell cycle stages [11–14].

The anaphase-promoting complex or cyclosome (APC/C) is an E3 ligase complex that promotes the ubiquitination and proteosomal degradation of substrates during mitotic exit [15]. APC/C substrate recognition is regulated by cofactors such as cell division cycle 20 (CDC20) and CDC20 homologue 1 (CDH1). During mitosis, APC/C^{CDC20} activity is inhibited by the spindle checkpoint. When the checkpoint is satisfied, inhibition of APC/C^{CDC20} is relieved and substrates are ubiquitinated and degraded. Early substrates such as securin and cyclin B1 are targeted at this time, whereas later substrates are degraded via APC/C^{CDH1} [15]. Both CDC20 and CDH1 recruit substrates to APC/C by recognizing degradation motifs within target proteins, such as the D-box with a minimal consensus sequence of RXXL, recognized by both CDC20 and CDH1, and the KEN-box, a specific CDH1 recognition motif. A second mechanism of APC/C activation exists that does not rely on substrate recruitment but rather only on the activation of APC/C by CDC20 [16].

Here we show that during mitotic exit conductin is targeted for proteosomal degradation by APC/C^{CDC20}. The regulation of conductin levels during the cell cycle controls the balance between S33/37/T41 phosphorylated (phospho) β -catenin and dephosphorylated (activated) β -catenin, and thus regulates β -catenin-mediated transcription in a cell cycle-dependent manner.

RESULTS AND DISCUSSION

Conductin levels are regulated during mitotic exit

To dissect the timing of conductin expression during the cell cycle, we analysed protein levels in SW480 and DLD1 colon cancer cells after release from G1 phase. Conductin levels reached a peak at G2/M, thereafter declining as mitotic cells returned to G1, whereas the related protein axin remained constant (Fig 1A; supplementary Fig S1A online). Moreover, conductin levels were higher in mitotic as compared with G1

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Fig 1 | Conductin levels oscillate during the cell cycle. (A) Western blotting of indicated proteins in lysates from SW480 cells collected at indicated hours (h) after release from mimosin synchronization. Numbers below show the percentage of cells in G1, S and G2/M stages at each time point. (B) Western blotting of conductin and β-actin in lysates of Rat2-Wnt1 cells collected at indicated time points after release from aphidicolin synchronization. (C) Western blotting of SW480 cell lysates at indicated time points after release from nocodazole-induced mitotic arrest. (D) Western blotting of conductin and β-actin from SW480 cell lysates collected at indicated time points after release from mitotic arrest in the presence of proteasome inhibitor (ALLN) or control derivative (ALLM), added 30 min after release from mitotic arrest. In all panels, cell cycle stages are indicated on top. APC, adenomatous polyposis coli; CDC20, cell division cycle 20.

phase 293T fibroblasts (supplementary Fig S1B online). In Rat2 cells stably expressing Wnt1 (Rat2-Wnt1), conductin levels increased reaching a peak at G2/M, thereafter being reduced (Fig 1B). As Rat2-Wnt1 cells exhibit constitutive Wnt signalling, changes in conductin levels during the cell cycle are unlikely to be due to Wnt signalling. Conductin levels drastically decreased within 1–2 h after mitotic exit, whereas total β-catenin levels remained constant (Fig 1C). Proteasomal inhibition blocked the diminishing conductin levels, suggesting that conductin is actively degraded during mitotic exit (Fig 1D). Inhibition of protein translation in asynchronous cells indicated a 6-h half-life for conductin (supplementary Fig S1C online). In contrast, during mitotic exit, conductin has a half-life of less than an hour (Fig 1C), indicating an active protein turnover during this period of the cell cycle. A tankyrase inhibitor (XAV939), which blocks polyubiquitination and degradation of conductin stabilized the protein in most phases of the cell cycle, suggesting that XAV939 does not act at a specific cell cycle stage and in particular is not involved in establishing the oscillatory pattern of conductin during the cell cycle (supplementary Fig S1D online) [17].

Cell cycle control of activated β-catenin by conductin

We noticed in Fig 1A that phospho-β-catenin closely followed the increase in conductin during G1 to G2/M progression, whereas

total β-catenin levels remained unchanged. Transient or stable knockdown of conductin by small interfering RNA (siRNA) or inhibition of GSK3β by LiCl blocked the increase in phospho-β-catenin in S and mitotic phases, suggesting that conductin/GSK3β complexes phosphorylate β-catenin during the cell cycle (Fig 2A,B, supplementary Fig S1E online). Levels of dephosphorylated activated β-catenin reduced as cells advanced to G2/M (Fig 2C). Importantly, during mitotic exit and progression to G1/S, degradation of conductin was followed by a decrease in phospho-β-catenin and a concurrent increase in activated β-catenin (Fig 2D). In APC-mutated SW480 cells used here, the β-catenin degradation complex can still lead to β-catenin phosphorylation, albeit not to efficient degradation [18]. Previous studies have indicated that phosphorylated β-catenin is transcriptionally inactive, whereas N-terminally dephosphorylated β-catenin is the active species increased by Wnt signalling [19,20]. The results therefore suggest that oscillation of conductin levels during the cell cycle of colon cancer cells determines the balance between phosphorylated and activated β-catenin, and thus inactive and active Wnt signalling. Indeed, reverse transcription-PCR analysis showed that Wnt/β-catenin target genes (c-Myc, Nr-Cam, FGF18 and SGK1) were significantly downregulated at G2/M (Fig 2E). There were two exceptions: stem cell marker LGR5 and conductin itself were upregulated at G2/M. At present, this

Fig 2 | Conductin regulates the ratio of phosphorylated and activated β -catenin during the cell cycle. (A) Western blotting for conductin, phosphorylated β -catenin, ABC, cyclin B1 and β -actin in lysates from SW480 cells transfected with siRNAs against conductin (siCond) or luciferase (siLuc) collected at indicated time points after release from aphidicolin synchronization. (B) Western blotting as in A of lysates from SW480 cells at indicated time points after release from aphidicolin synchronization in media containing LiCl or NaCl as control. (C) Western blotting for indicated proteins in lysates of synchronized SW480 cells during progression from G1 to mitosis (M). (D) Western blotting for conductin, phosphorylated β -catenin, ABC, cyclin B1 and β -actin in lysates from SW480 cells collected at indicated time points after release from nocodazole-induced mitotic arrest. (E) RT-PCR for indicated target genes at 3 (G1/S) and 18 (G2/M) hours after release from mimosin synchronization. (F) TOP/FOP luciferase activities in SW480 cells synchronized at mitosis (M) and after progression through G1 (G1/S) for indicated times. Asterisks show statistically significant differences from the 0-h time point ($P < 0.05$). In all panels cell cycle stages are indicated. ABC, activated β -catenin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LiCl, lithium chloride; NaCl, sodium chloride; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA.

remains unresolved, but previous studies indicate that conductin is a target gene of E2F transcription factors and LGR5 is a target gene of c-Jun/Mbd3 whose activation is high at G2/M [21,22]. Moreover, the activity of TOP/FOPFlash luciferase reporters carrying TCF/ β -catenin-responsive DNA elements increased as cells progressed from mitosis to G1/S, in line with target gene expression and in support of the data showing increase in activated β -catenin during this cell cycle period (Fig 2F).

Conductin protein stability is regulated by CDC20

The sharp decrease in conductin after mitosis (Fig 2D) is reminiscent of APC/C^{CDC20}-mediated degradation of early substrates. Ectopic expression of CDC20 in asynchronous cells activates APC/C and targets substrates for degradation [23]. In

coexpression assays, green fluorescent protein (GFP)-CDC20 reduced Flag-conductin levels in a proteasome-dependent manner, whereas GFP did not (Fig 3A). CDC20 can mediate degradation of substrates either by recruiting them to APC/C, via its WD40 repeats, or only by activating APC/C towards substrates such as Nek2, which directly bind to APC/C subunits via their isoleucine/arginine (IR) domain (supplementary Fig S2A online). A mutant of CDC20 (N159) can activate APC/C but lacks substrate recruitment capabilities [16]. Whereas increasing amounts of GFP-CDC20 mediated degradation of conductin, GFP-N159 did not, although GFP-N159 induced APC/C-mediated degradation of Nek2 as previously reported (Fig 3B; supplementary Fig S2B online). GFP-CDH1, which can also activate APC/C, did not affect Flag-conductin levels and did not coimmunoprecipitate

Fig 3 | CDC20 interacts with conductin and induces its proteosomal degradation. (A) WB of lysates from 293T cells co-transfected with Flag-conductin alone or with GFP-CDC20 and treated with proteosomal inhibitors MG132 and ALLN or control ALLM for 1 h before lysis. (B) WB of lysates from 293T cells co-transfected with Flag-conductin and increasing amounts of GFP-CDC20, a CDC20 mutant lacking the WD40 repeats (GFP-N-159), or GFP-CDH1 as indicated. GFP plasmid was used to keep total DNA amounts equal. (C) WB for HA-Ub and Flag-expressed proteins after Flag IP from lysates of 293T cells transfected with indicated plasmids in the presence or absence of proteosomal inhibitor MG132. Ubiquitin chains (HA-Ub), as well as antibody heavy/light chains (asterisks), are indicated. (D) WB for endogenous conductin and CDC20 after IP with antibodies against conductin, CDC20 or control (IgG) from lysates of mitotic SW480. (E) Western blotting for endogenous conductin, CDC20 and β -actin in lysates of SW480 and HCT116 colon cancer cells, transfected with siRNAs against CDC20 or GFP, 1 h after release from nocodazole-induced mitotic arrest. CDC20, cell division cycle 20; GFP, green fluorescent protein; HA-Ub, HA-Ubiquitin; IP, immunoprecipitation; WB, western blot.

Flag-conductin (Fig 3B and data not shown). In addition, axin was not degraded by GFP-CDC20, in line with its constant expression during the cell cycle (supplementary Fig S2C online). Expression of GFP-CDC20 stimulated ubiquitination of coexpressed Flag-conductin in the presence of HA-Ubiquitin, which increased after proteosomal inhibition (Fig 3C, lanes 3 and 4, respectively), but not of Flag-TACC3 used as a control protein (Fig 3C, lane 6). Consistent with our findings, GFP-CDC20 coimmunoprecipitated Flag-conductin, whereas GFP-N159 could not (supplementary Fig S2D online). Importantly, endogenous conductin/CDC20 complexes were immunoprecipitated from mitotic cells (Fig 3D). Taken together, these findings suggest that CDC20, but not CDH1, targets conductin for ubiquitination and degradation. In line with this, siRNA-mediated knockdown of CDC20 led to stabilization of conductin in SW480 and HCT116 cells, 1 h after mitotic exit, and in asynchronous cells, but did not influence axin protein levels (Fig 3E; supplementary Figs S2E and S5C online).

Out of four putative D-boxes, known to mediate degradation by CDC20, only D-box1 is conserved also in zebrafish and

Xenopus conductin proteins (Fig 4A). We generated single and compound mutants (Flag D1–D4) by substituting arginine and lysine residues with alanine, and assessed degradation by CDC20. Whereas single mutants Flag-D2, -D3, -D4 were degraded by GFP-CDC20, Flag-D1 and compound mutants Flag-D134 and Flag-D1234 were resistant (Fig 4B). The conserved D-box1 might therefore be a functional CDC20 degradation motif. Indeed, immunoprecipitation experiments indicated that D-box mutant conductin binds weakly to CDC20 (Fig 4C). Collectively, the results suggest that conductin is a bona fide substrate for CDC20-mediated degradation during mitotic exit.

CDC20 regulates Wnt/ β -catenin signalling via conductin

To analyse whether activation of APC/C by CDC20 influences Wnt/ β -catenin signalling, we assessed the activity of TOP/FOPFlash reporters in mitotic SW480 cells after coexpression of GFP-CDC20. CDC20 increased TOP/FOP activity as compared with control GFP transfection (Fig 5A). Reciprocally, knockdown of CDC20 reduced reporter activity in G1 cells and concurrent

Fig 4 | CDC20 mediates degradation of conductin via a conserved degradation domain. (A) Schematic representation of mouse conductin protein and interaction domains for Wnt-signalling components, as well as putative D-boxes. Below, alignment of putative D-boxes (in bold) and surrounding amino acids is shown for human, mouse, zebrafish and *Xenopus* sequences. Asterisks indicate conservation. (B) WB of lysates from 293T cells co-transfected with single D-box mutants of Flag-conductin (Flag-D1, -D2, -D3, -D4), as well as compound mutants (Flag-D134, Flag-D1234) together with GFP or GFP-CDC20 (arrowheads). (C) WB for GFP and Flag after IP with a GFP antibody from lysates of 293T cells co-transfected with indicated plasmids. Expression of Flag-tagged constructs in lysates is shown in lower panel (INPUT). CDC20, cell division cycle 20; GFP, green fluorescent protein; IP, immunoprecipitation; WB, western blot.

knockdown of conductin blocked this effect, suggesting that during the cell cycle CDC20 regulates Wnt/β-catenin signalling through conductin (Fig 5B). Knockdown of CDC20 in asynchronous HCT116 cells also decreased reporter activity (supplementary Fig S2F online). We presume that in HCT116 cells conductin acts mainly by cytoplasmic retention of mutated β-catenin [24]. Importantly, knockdown of CDC20, which led to increased conductin levels and β-catenin phosphorylation, reduced expression of all β-catenin target genes tested, whereas concurrent knockdown of conductin, which increased activated β-catenin, alleviated the reduction in target gene expression (Figs 5C,D). Overexpression of Flag-conductin in SW480 cells reduced TOP/FOP reporters, and coexpression of GFP-CDC20 counteracted this effect (Fig 5E). Importantly, GFP-CDC20 could not counteract the reduction of TOP/FOP in response to coexpressed CDC20-resistant mutant Flag-D1 (Fig 5E). We next assessed the ability of wild-type, as well as CDC20-resistant, conductin to inhibit proliferation of colon cancer cells. Expression of Flag-D1 mutant, but not of wild-type Flag-conductin or Flag-D2, significantly inhibited colony formation of SW480 cells but did not affect that of human osteosarcoma (U2OS) cells, which do not rely on aberrant Wnt signalling for cell growth (Fig 5F,G). Transfection efficiencies were similar for all plasmids (about 33% for SW480 and 40% for U2OS cells). Our data suggest that CDC20 regulates Wnt/β-catenin signalling and growth of colon cancer cells by controlling protein levels of conductin during the cell cycle.

Several studies have shown that β-catenin levels increase during G1 to G2/M progression in several cell lines, reviewed in Davidson and Niehrs[11]. These findings seem to contradict our

results. However, these studies were mainly performed in Wnt signalling-independent cells, as well as non-transformed cells, or after brief stimulation with Wnts to activate β-catenin-dependent transcription. Thus, they did not invoke a conductin-dependent feedback mechanism, which becomes relevant in cells with chronic aberrant Wnt signalling.

APC/C activity is crucial for mitotic exit, and transition to G1 is a key event for cell proliferation because it determines whether, or not, cells will commit into a new round of duplication. It is plausible that proliferative pathways such as Wnt/β-catenin signalling show peak activity during G1. In line with this, we found high levels of activated β-catenin and low levels of phospho-β-catenin in G1 cells, which are dependent on conductin, suggesting that the low abundance of conductin during G1 allows Wnt signalling activation and cell cycle progression. Moreover, high levels of conductin during mitosis restrict the dosage of Wnt signalling, which might account for a reduction in transcription of genes that would otherwise be deleterious for progression through G2/M. Importantly, our finding that the low abundance of conductin after mitotic exit is established by APC/C^{CDC20} suggests a molecular mechanism that resets the cell cycle clock by coupling mitotic events to proper activation of a signalling pathway in subsequent cell cycle stages. In most colorectal tumours, conductin expression is high but apparently not sufficient to inhibit Wnt signalling [9,13]. Our results show that conductin can regulate GSK3β-mediated phosphorylation of β-catenin in colorectal cancer cells. We suggest that Wnt signalling escapes inhibition by conductin at a critical cell cycle stage (G1/S) because of CDC20-mediated

Fig 5 | CDC20 regulates Wnt signalling through conductin. TOP/FOP ratios of luciferase activities in SW480 cells transfected with reporters and GFP-CDC20, or GFP, collected 9 h after release from aphidicolin synchronization (G2/M) (A), or with indicated siRNAs collected 9 h after release from nocodazole arrest (G1/S) (B). (C) Western blotting for endogenous proteins in lysates of SW480 cells transfected with indicated combinations of siRNAs against GFP, CDC20 and conductin. (D) RT-PCR for indicated target genes in cells from C. (E) TOP/FOP ratios of luciferase activities in SW480 cells transfected with reporters and indicated combinations of expression plasmids. Asterisks indicate statistically significant differences from control (GFP; $P < 0.05$). (F) Fluorescence images of SW480 cell colonies stained with ethidium bromide after transfection with indicated plasmids, seeded at cell numbers shown on top. Automated colony counts are shown within images. (G) Quantification of F and of similar experiments in U2OS cells. Values are percentages of number of colonies in Flag-conductin-transfected wells. Error bars show standard error from at least four experiments. * $P < 0.05$. CDC20, cell division cycle 20; Cond, conductin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; RT-PCR, reverse transcription-PCR; siRNA, small interfering RNA.

degradation of conductin during mitotic exit. Indeed, a CDC20-resistant mutant of conductin was stronger in blocking Wnt signalling and proliferation of colon cancer cells than wild-type conductin. Three recent independent screens for small-molecule inhibitors of Wnt signalling and cancer cells have identified conductin stabilization as the underlying mechanism [17,25,26]. Thus, stabilization of conductin might represent cancer's Achilles' heel. We propose that uncoupling conductin from cell cycle control, for instance by inhibiting its APC^{CDC20}-mediated degradation, could constitute a feasible way of using high conductin levels in colorectal cancers to selectively inhibit Wnt/ β -catenin signalling and proliferation of cells.

METHODS

Cell culture, synchronization and transfections. Colorectal cancer cell lines SW480, HCT116, DLD1, human embryonic kidney fibroblasts HEK293T (293T), Rat2-Wnt1 fibroblasts and human osteosarcoma U2OS cells were grown in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C/10% CO₂. Nocodazole (0.2 µg/µl), aphidicolin (1 µg/µl), mimosin (0.5 mM), cycloheximide (100 µg/ml), MG132 (20 µM), N-acetyl-Leu-Leu-Norleu-al (ALLN) (25 µM) and N-acetyl-Leu-Leu-Met-CHO (ALLM) (25 µM) were purchased from Sigma; LiCl (30 mM) and NaCl (30 mM) from Roth and XAV939 (2 µM) from Maybridge. For G1 synchronization, cells were either starved for 24 h followed by addition of mimosin for an additional 24 h, or incubated with aphidicolin for 24 h. For synchronization in mitosis, cells were treated with nocodazole for 18 h and mitotic cells were isolated after selective shake-off. Previously published siRNAs against GFP [27], conductin [12], as well as against CDC20 (sc-36160; Santa Cruz), were transfected with oligofectamin (Invitrogen, Carlsbad, CA, USA). Plasmids were transfected with polyethylenimine. Co-transfections of siRNAs and plasmids were performed with Lipofectamine 2000 (Invitrogen).

Molecular biology. Standard molecular biology methods were used for cloning N-terminal GFP- and Flag-tagged constructs. Point mutants of conductin (D1,2,3,4) were generated using the Quickchange Site-Directed mutagenesis kit (Stratagene). Human CDC20 and CDH1 were cloned from 293T complementary DNAs (cDNAs). Conductin, axin1 and Nek2 constructs have been described [12]. For reverse transcription-PCR, total RNA was prepared using the RNeasy Mini Kit (Quiagen), and cDNAs were generated from 1 µg of RNA with the AffinityScript QPCR cDNA Synthesis Kit (Stratagene). Primer sequences for PCR are available in supplementary information online.

Biochemistry and antibodies. Western blotting was performed as described in Dehner et al [27]. Primary antibodies rabbit anti-axin1, anti-phospho- β -catenin (Ser33/37/Thr41), mouse anti-HA (Cell Signalling), mouse anti-active- β -catenin (anti-ABC; Millipore), mouse anti-Flag, mouse anti- β -actin (Sigma), mouse anti-GFP (Roche), mouse anti-APC (Ali12-28; Abcam), goat anti-p53 CDC20 (C-19), rabbit anti- β -catenin (H102; Santa Cruz) and mouse anti-Cyclin B1 (Upstate) were used according to the manufacturer's instructions. For detection of conductin, the mouse C/G7 antibody was used [9]. Immunoprecipitations were performed as described in Hadjihannas et al [12].

TOP/FOPFlash assays. Cells transfected with TOP/FOPFlash reporters and plasmids for 24 h were synchronized as indicated in the manuscript and luciferase activity measured as described in Dehner et al [27].

Colony formation assay. Cells were transfected with plasmids for 24 h. Transfection efficiency was determined and cells were trypsinized, counted and seeded at 2,000, 3,000, 4,000 and 6,000 cells per well. The medium was replenished every 3 days until colony formation was observed. Colonies stained in a solution of ethidium bromide in 50% ethanol were imaged in a UV transilluminator equipped with a Gerolab CCD camera. The number of colonies was determined using the Metamorph software and the Integrated Morphometry Analysis module.

Statistical analyses and calculation of *P* values were performed using Student's *t*-test.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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