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The Wnt Pathway Target Gene *CCND1* Changes Mitochondrial Localization and Decreases Mitochondrial Activity in Colorectal Cancer Cell Line SW480

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Abstract

Mutations leading to constitutive activation of the Wnt pathway and its target genes are frequently observed in cancer. The Wnt pathway promotes cell proliferation and increasing evidence supports its role also in cancer cell metabolism. This study aims to elucidate the role of the Wnt/β-catenin target gene *CCND1* in these processes in colorectal cancer. We analyzed whether knock-down of *CCND1* affects cell cycle progression and energy metabolism in a colorectal cancer cell line. Down-regulation of *CCND1* led to retardation of the cell cycle. The proportion of cells in the G0 phase increased, while the amount of cells in the S- and G2/M phase decreased. Interestingly, knock-down of *CCND1* changed the perinuclear localization of mitochondria into a homogeneous distribution within the cytosol. In addition *CCND1* knock-down led to an increase of the intracellular ATP level indicating that cyclin D1 reduced mitochondrial activity. Our findings suggest that in addition to its role in cell cycle regulation, the Wnt target gene *CCND1* regulates mitochondrial localization and inhibits mitochondrial activity in colorectal cancer cells.

Keywords

ATP, Cyclin D1, Colorectal Cancer, Mitochondria, Wnt

1. Introduction

The Wnt pathway controls several different cellular processes and mutations in com-

ponents of the Wnt pathway are frequently found in solid cancers [1]. Besides other features, the Wnt pathway regulates pluripotency in stem cells and cancer stem cells [2]. Key event of the canonical Wnt pathway is the regulation of the level of the free proto-oncoprotein β-catenin which is encoded by *CTNNB1*. In a cell with an inactive Wnt pathway, the β-catenin level is kept low, whereas in the Wnt-activated cell β-catenin is stabilized, accumulates in the cytoplasm and translocates into the nucleus. Nuclear β-catenin forms a complex with transcription factors of the T-cell factor/Lymphoid enhancer factor (TCF/LEF) family, which activates transcription of many different target genes [3] [4]. A detailed list of the Wnt target genes can be found on the Wnt homepage [5].

The gene *CCND1* codes for the cyclin dependent kinase (cdk) activator cyclin D1. *CCND1* has been described as a Wnt target gene in different systems, e.g. human colorectal cancer cells [6] [7] and human teratocarcinoma cells [8]. However, these results could not be confirmed in other studies [9] [10] [11]. Nevertheless, in cell lines *CCND1* can be activated upon extracellular stimulation of the canonical Wnt pathway and belongs to the early target genes [12]. In complex with cdk4 or cdk6 cyclin D1 activates G1/S phase transition [13] [14] [15]. In addition to its function in cell cycle progression, cyclin D1 has emerging roles in mitochondrial metabolism [16]. Wnt signalling is known to be involved in metabolic homeostasis in normal cells [17] and recent data also support a role for the Wnt pathway in cancer cell metabolism since Pate *et al.* reported that Wnt signalling reduces glycolytic metabolism in colon cancer cells [18]. Until now the extent to which *CCND1*—one of the main Wnt target genes—is able to influence mitochondrial metabolism in colorectal cancer cells has remained unknown.

Therefore, we investigated in this study the impact of cyclin D1 on cell cycle distribution and mitochondrial activity in a colorectal cancer cell line harboring a constitutive active Wnt pathway. We demonstrate here that *CCND1* is one of the Wnt targets that is not only involved in proliferation, but also in metabolic reprogramming of colorectal cancer cells.

2. Materials and Methods

2.1. Cell Lines and siRNA Transfection

In the human colorectal carcinoma cell line SW480 the Wnt pathway is constitutively activated and the β-catenin level is increased due to a lack of full-length APC protein [19]. SW480 cells were obtained from the DSMZ cell line bank (Braunschweig, Germany) and were cultured at 37˚C and 7.5% CO_2 in a humidified atmosphere in DMEM (Invitrogen, Karlsruhe, Germany) supplemented with 10% (v/v) fetal calf serum, penicillin (2.85 U/ml) and streptomycin (2.85 µg/ml), L-glutamine (11.4 mM), non-essential amino acids (0.57 mM) and sodium pyruvate (11 nM). siRNA was purchased from Dharmacon (distributed by Perbio, Bonn, Germany). Among these siRNAs were cyclin D1 siGENOME SMARTpool, the β-catenin siGENOME SMARTpool and GAPDH siGENOME SMARTpool. As a control siCONTROL non-targeting #1 was used. Cells were transfected using the transfection reagent DharmaFECT 1 (Dharmacon) according

to the manufacturer's instructions.

2.2. Purification and Reverse Transcription of RNA

Purification of RNA was performed using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and following the supplied protocol. Reverse transcription of 1.5 µg total RNA was performed in a total volume of 20 µl 1× RT buffer containing 1 µl Omniscript RT (Qiagen), 1 µM oligo-dT primer, 1 U RNaseOut and 500 µM dNTP by incubation for 60 min at 37˚C followed by an incubation for 5 min at 93˚C.

2.3. Quantitative Real-Time PCR

Quantitative real-time PCR (qRT-PCR) was performed using QuantiTect SYBR Green PCR Kit (Qiagen) following the protocol of the manufacturer. Each reaction was done in triplicates using the GeneAmp 5700 Sequence Detection System (Applied Biosystems, Carlsbad, USA). Data were evaluated with the GeneAmp 5700 SDS software, *ACTB* was used as endogenous control. The sequences of the primers used for qRT-PCR are for *ACTB* 5'-gtcttcccctccatcgtgggg-3' (forward) and 5'-gtgaggatgcctctcttgctctg-3' (reverse), for *CTNNB1* 5'-gatcttggacttgatattggtgcccag-3' (forward) and 5'-tccatacccaaggcatcctggcc-3' (reverse), and for *CCND1* 5'-gcctgaacctgaggagcccca-3' (forward) and 5'-gtcacacttgatcactctgg-3' (reverse).

2.4. Immunocytochemistry and Fluorescence Microscopy

Staining of mitochondria was performed with the dye MitoTracker® Green FM from Molecular Probes (Invitrogen, Karlsruhe, Germany). Cells were cultivated on glass slides, stained with 200 nM MitoTracker® Green FM in DMSO for 45 min at 37˚C and fixed for 20 min with formaldehyde (3.7% in PBS). Next, cells were washed with medium, permeabilized in cold acetone/methanol (1:1) for 5 min at −20˚C and air-dried for 10 min at room temperature. Cell nuclei were counterstained in 0.05 µg/ml DAPI (4,6-Diamidino-2-phenylindole) in PBS for 5 min, washed once with PBS and mounted on slides with fluorescent mounting medium (Dako, Glostrup, Denmark). Analysis and evaluation were performed with the microscope Axiophot (Zeiss, Oberkochen, Germany) and the software Axiovision Release 4.6.3.

2.5. Flow Cytometry

Staining of the cells with MitoTracker® Green FM was performed as described above for fluorescence microscopy. After harvesting the cells were centrifuged for 5 min at 1500 rpm and resuspended in PBS. Fixation and permeabilization of 1×10^6 cells were performed with commercial reagents (BD Cytofix/Cytoperm Fixation/Permeabilization Kit from Becton Dickinson, Heidelberg, Germany) by following the protocol of the manufacturer. The cells were analyzed by FACS (LSRII from BD) and data were evaluated with the software FlowJo (TreeStar Inc., Ashland, USA).

Proportion of cells in different cell cycle phases were determined by propidium iodide staining and flow cytometry. After harvesting the cells were centrifuged for 5 min

at 1500 rpm and resuspended in PBS. One million cells were resuspended in 500 µl lysis buffer including 100 ng/µl RNase and 10 ng/µl propidium iodide (PI) and incubated for 1 h at 4˚C. Next cells were analyzed by FACS (FACS Calibur from BD) and data were evaluated with the software CellQuestPro (BD).

2.6. Protein Gel Electrophoresis and Western Blot

Cell lysates were electrophoresed in a vertical mini-system (BioRad, München, Germany) using standard buffers. Proteins were transferred on a PVDF membrane (GE Healthcare, München, Germany (GE)) in a semi-dry cell (Biometra, Göttingen, Germany). The membrane was blocked in 5% milk powder in PBS/T (PBS with 0.5% Tween20). Next, the primary antibody was added at a specific dilution. Anti-cyclinD1 antibody (Santa Cruz Biotechnology, Heidelberg, Germany) was used at a 1:500 dilution, anti-β-actin (Abcam, Cambridge, UK) was used at 1:20,000. The membrane was washed in PBS/T before adding the secondary HRPO-coupled antibody (GE) at a dilution of 1:10,000. Proteins were detected by enhanced chemiluminescence (ECL) using ECL Western blotting substrate kit (Abcam, Berlin, Germany) and Hyperfilm ECL (GE).

2.7. Analysis of Cellular ATP Level

Total cellular ATP level was measured using CellTiter-Glo® Luminescent Cell Viability Assay (Promega, Mannheim, Germany) according to the manufacturer's instructions. Twenty-four hours prior to ATP measurements SW480 cells were transfected with siRNAs against *CCND1* (*CCND1*_5 SI02654540, *CCND1*_6 SI02654547 in a molar ratio 1:1, Qiagen) and AllStars Negative Control siRNA as control (ID 1027280, Qiagen) using HiPerFect Transfection Reagent (Qiagen). In order to determine the relative amount of total ATP per cell, cells were harvested and counted by live-dead staining with trypan blue (Sigma, München, Germany) in a hemocytometer. Samples were measured in triplicates in 96-well plates using 50,000 viable cells per well against a standard curve with ATP (Roth, Karlsruhe, Germany) using CellTiter-Glo Reagent in a microplate reader (TECAN infinite M200, Tecan Group, Männedorf, Switzerland).

3. Results

3.1. Cyclin D1 Is a Wnt Target Gene in SW480 Cells

Because only a few Wnt target genes are universal across cell types [3], *CCND1* had to be confirmed as Wnt/β-catenin target gene in the chosen model cell line. In this study we used the colorectal cancer cell line SW480 that harbors a constitutively active canonical Wnt pathway. Therefore, we inactivated the Wnt pathway by decreasing β-catenin levels with siRNA in SW480 cells. **Figure 1(a)** shows the decrease of the relative *CTNNB1* level of more than 80% 24, 48 and 72 h after transfection. The *CCND1* mRNA level in β-catenin siRNA treated SW480 cells decreased by 30% (**Figure 1(b)**). Therefore, *CCND1* expression depends on β-catenin and *CCND1* could be confirmed as Wnt target gene in SW480 cells.

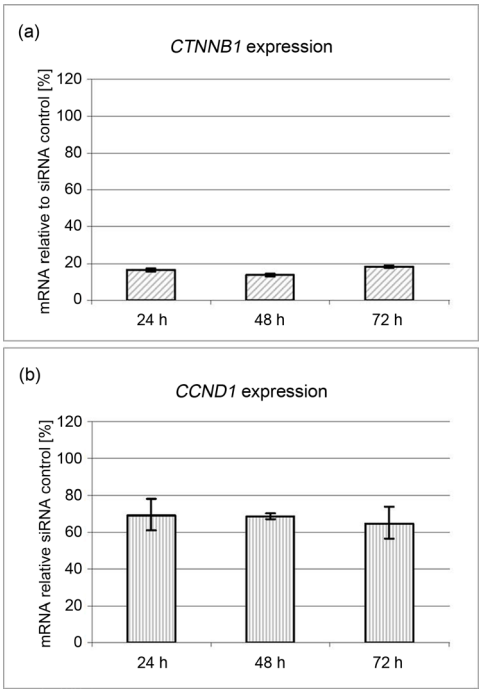

Figure 1. *CCND1* expression depends on β-catenin in SW480 cells. Transfection of *CTNNB1* siRNA into SW480 cells downregulates expression levels of *CTNNB1* mRNA (a) and effects a reduced expression of *CCND1* (b) in comparison to cells transfected with control siRNA. Expression levels were analyzed by qRT-PCR at 24, 48 and 72 h after transfection of *CTNNB1* siRNA or control siRNA. At each time point expression level in control siRNA treated cells was set at 100%. n = 2.

3.2. Cyclin D1 Promotes G1/S Transition in SW480 Cells

Next, we tested the influence of cyclin D1 on cell cycle progression. Therefore, SW480 cells were transfected with siRNA against *CCND1* or a non-silencing control siRNA and knock-down was verified at the mRNA level (**Figure 2(a)**) and the protein level (**Figure 2(b)**). Analysis of the relative amounts of cells in different phases of the cell cycle was performed using propidium iodide staining and flow cytometry (**Figure 2(c)**). Down-regulation of *CCND1* leads to an increase of the relative cell number in the G0/G1 phase and to a decrease of the relative cell number in the S- and G_2/M phase, respectively. Thus, cyclin D1 promotes proliferation of SW480 cells.

3.3. Cyclin D1 Alters Mitochondrial Distribution and Activity in SW480 Cells

Mitochondrial distribution and mass was analyzed in *CCND1* siRNA treated cells to determine the role of cyclin D1 in this process. The mitochondria were visualized with MitoTracker® Green FM, which specifically intercalates into the mitochondrial membrane, 48 h post transfection of siRNA against *CCND1* or non-silencing control siRNA. Interestingly, the perinuclear mitochondrial distribution was abolished in cells upon *CCND1* knock-down leading to a homogenous distribution of mitochondria within the cytosol (**Figure 3(a)**). In addition, flow cytometry showed that the fluorescent signal of

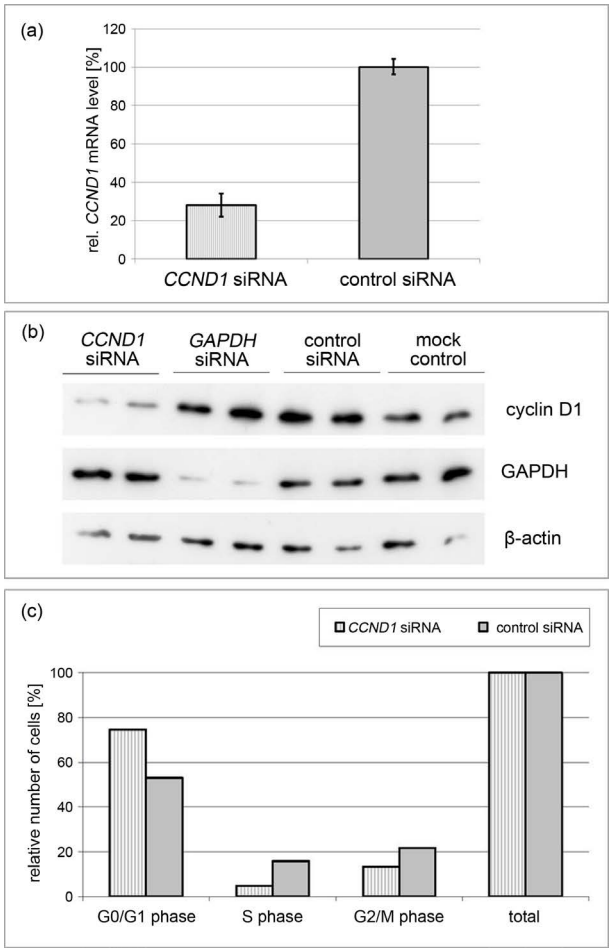

Figure 2. Knock-down of *CCND1* in SW480 cells alters cell cycle distribution. (a) qRT-PCR shows relative mRNA level of *CCND1* after transfection of siRNA against *CCND1*. The mRNA level in non-targeting siRNA treated cells was set at 100%. n = 3. (b) Effect of *CCND1* knock-down on cyclin D1 protein level as shown by a representative Western Blot 48 h post transfection. *GAPDH* siRNA transfection served as positive control. All transfections were performed in duplicates. (c) The relative amount of cells in the G0/G1-, S- and G2/M phase compared to non-silencing control siRNA treated cells 48 h after transfection. n = 2.

mitochondria was increased in cells with knocked-down *CCND1* when compared to control cells (Figure 3(b) and Figure 3(c)), indicating that cyclin D1 affects the mitochondrial mass in the cells.

In order to test if cyclin D1 also influences mitochondrial activity, we next investigated the intracellular ATP level. Again SW480 cells were transfected with siRNA against *CCND1* or non-silencing siRNA as control, and the cellular ATP levels were determined 24 h post transfection with an ATP assay that is based on the luminescence released during an ATP-dependent luciferase reaction. After *CCND1* knock-down the relative cellular ATP level increased 1.5 fold in comparison to the control (Figure 3(d)). Expression analysis of *CCND1* in these samples confirmed that *CCND1* mRNA level significantly decreased to 40% after siRNA transfection (Figure 3(e)). Thus, cyclin D1 does not only influence the mitochondrial mass in SW480 cells, but also the mitochondrial

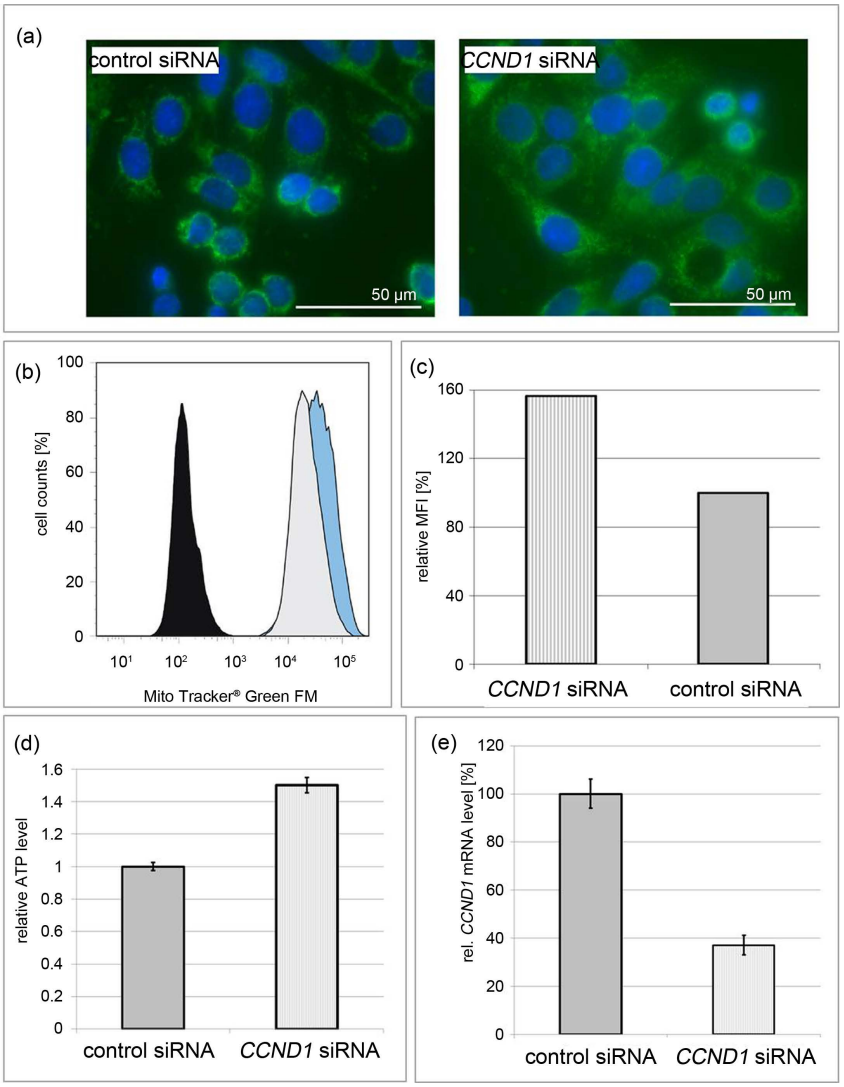

Figure 3. *CCND1* regulates mitochondrial distribution and cellular ATP levels in SW480 cells. (a) Immunofluorescences of *CCND1* siRNA or control siRNA treated cells after MitoTracker® Green FM (green) staining. Nuclei of SW480 cells were counterstained with DAPI (blue). (b) Flow cytometry analysis after MitoTracker® Green FM staining. Representative histograms for cells transfected with siRNA against *CCND1* (blue) or non-silencing control siRNA (grey) in comparison to untreated and unstained cells (black) 48 h post transfection. (c) Quantification of the relative mean fluorescence intensity (MFI) of MitoTracker® Green FM as shown in (b). (d) Relative total cellular ATP levels determined 24 h after transfection of siRNA against *CCND1* or non-targeting siRNA as control. The ATP amount in the control was set at 1. Error bars indicate standard deviation of technical triplicates. (e) The results of the qRT-PCR show the relative mRNA level of *CCND1* to control within the samples shown in (d). Shown are representative data of one of three biological replicates.

activity as verified by the increase of the cellular ATP-level after *CCND1* knock-down.

4. Discussion

In this study, we investigated to which extent cyclin D1 as one of the major Wnt targets

is responsible for mediating downstream effects on proliferation and metabolism of constitutively active Wnt signaling in colorectal cancer cells. In accordance with previous studies [6], we confirmed *CCND1* as a canonical Wnt target gene in the colorectal cancer cell line SW480. Like most human colon cancers, this cell line has no functional APC protein, thus resulting in the accumulation of *β*-catenin. Knock-down of *β*-catenin in SW480 led to a decrease in *CCND1* transcript level (**Figure 1**). In order to investigate the impact of the Wnt target cyclin D1 on proliferation, cell cycle progression was investigated by propidium iodide staining and flow cytometry. As expected, a cell cycle arrest in the G1 phase was observed upon knock-down of *CCND1* (**Figure 2**). The results indicate that proliferation, specifically the G1/S transition during cell cycle progression, of SW480 cells is significantly regulated by *CCND1*. This conclusion is in agreement with other studies showing that cyclin D1 is important for G1/S progression [20]. In addition to its classical function in cell cycle progression, cyclin D1 is known to regulate mitochondrial metabolism [16]. For example it was shown in mice that cyclin D1 deficiency increases mitochondrial size and activity [21].

Mitochondria play an important role in different cellular processes including growth, division, energy metabolism and apoptosis [22] [23]. They are dynamic structures that merge and divide. The mitochondrial network is continuously rearranged [24]. Importantly, the mitochondrial energy production influences cellular proliferation and tumor progression. An altered energy metabolism is a hallmark of tumor cells [22]. In a normal cell, mitochondria are responsible for the production of more than 90% of the energy, predominantly by oxidative phosphorylation. In contrast, tumor cells produce a significant part of their energy by glycolysis, even under aerobic conditions. Pyruvate, the product of glycolysis, is metabolized to lactate, a process known as Warburg effect [25].

Although less ATP is produced during "aerobic glycolysis", this strategy provides a growth advantage for tumor cells [26]. Tumor cells adapt to hypoxic conditions and the fermentation of pyruvate to lactate, which leads to an acidification of the tumor microenvironment, facilitating tumor invasion [27] [28]. Consequently, the involvement of the mitochondria during tumorigenesis is not only limited to their well-known role in apoptosis, but they also have an influence on cellular proliferation and tumor progression [22].

In this study, we investigated the influence of the Wnt pathway target gene *CCND1* on distribution and activity of mitochondria as the role of cyclin D1 within these processes was unknown for colorectal cancers until now. Fluorescence microscopic images showed a wide and homogeneous distribution of the cell organelles in cells with knocked-down *CCND1*, in contrast to control cells that had a perinuclear distribution of mitochondria (**Figure 3**). In addition the mitochondrial mass was increased after *CCND1* knock-down. Furthermore, we could show that the high expression of *CCND1* in SW480 cells contributes to reduced total cellular ATP levels.

Our results show that cyclin D1 is involved in the extent of mitochondrial distribution and the reduction of the mitochondrial mass which correlated with the mitochon-

drial activity in the tested colon carcinoma cell line. These results are in agreement with the results of others obtained from other cell types. Sakamaki *et al.* have shown that cyclin D1 has an influence on the function and also on the size of mitochondria in breast cancer cells. They conclude that cyclin D1 promotes the glycolytic phenotype of mitochondria [29]. In addition, Tchakarska *et al.* described that cyclin D1 inhibits mitochondrial activity in B cells [30]. Cyclin D1 localizes to the outer mitochondrial membrane where it binds to a voltage-dependent anion channel and decreases the supply of ADP, ATP and metabolites, thereby reducing mitochondrial metabolism [30]. Cyclin D1 also controls glucose metabolism in hepatic cells by down-regulating PGC1*α*, thus suppressing gluconeogenesis and promoting the Warburg effect [31] [32]. Therefore, the high expression of cyclin D1 in colorectal cancer cells due to an active Wnt pathway might contribute to an altered cancer cell metabolism. After *CCND1* knock-down in SW480 cells we observed that the perinuclear distribution of mitochondria was abrogated. This indicates that cyclin D1 might support perinuclear clustering of mitochondria in colorectal cancer. Similar effects were reported for factors of the tumor necrosis factor family that are able to induce clusters of mitochondria around the nucleus [33]. Clustering of mitochondria in the perinuclear region is also triggered by hypoxia in endothelial cells which show under normoxia a homogenous cytoplasmic distribution of mitochondria [34]. Al-Mehdi *et al.* further showed that this results in higher nuclear ROS (reactive oxygen species) levels that are responsible for hypoxia-induced upregulation of vascular endothelial growth factor (VEGF). Thus, there are several examples in the literature, where the perinuclear localization of mitochondria is linked to cell death or stress. This connection does presumably not reflect the situation in the analyzed colorectal cancer cell line SW480 which loses its perinuclear clustering of mitochondria upon *CCND1* knock-down in parallel to the observed G1 arrest. Eventually, the finding that perinuclear distribution of mitochondria also correlates with pluripotency in stem cells [35] [36] enables the hypothesis that knock-down of *CCND1* rather reduces the pluripotent character of SW480 cells.

Thus, our data show that the Wnt target gene *CCND1* might be involved in three main effects which are performed by the Wnt pathway in colorectal cancer cells: it is not only crucial for G1/S transition during cell cycle progression, it also alters energy metabolism and it might affect the pluripotent character. Future investigations have to show, to which extent cyclin D1 fulfils these actions in colorectal carcinoma cells in general.

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