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# MCC inhibits beta-catenin transcriptional activity by sequestering DBC1 in the cytoplasm

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**The mutated in colorectal cancer (*MCC*) is a multifunctional gene showing loss of expression in colorectal and liver cancers. *MCC* mutations can drive colon carcinogenesis in the mouse and *in vitro* experiments suggest that loss of MCC function promotes cancer through several important cellular pathways. In particular, the MCC protein is known to regulate beta-catenin (β-cat) signaling, but the mechanism is poorly understood. Here we show that the β-cat repressor function of MCC is strongly impaired by the presence of a disease-associated mutation. We also identify deleted in breast cancer 1 (DBC1) as a new MCC interacting partner and regulator of β-cat signaling. RNA interference experiments show that DBC1 promotes β-cat transcriptional activity and that the presence of DBC1 is required for MCC-mediated β-cat repression. In contrast to all other DBC1 interacting partners, MCC does not interact through the DBC1 Leucine Zipper domain but with a glutamic-acid rich region located between the Nudix and EF-hand domains. Furthermore, MCC overexpression relocalizes DBC1 from the nucleus to the cytoplasm and reduces β-cat K49 acetylation. Treatment of cells with the SIRT1 inhibitor Nicotinamide reverses MCC-induced deacetylation of β-cat K49. These data suggest that the cytoplasmic MCC–DBC1 interaction sequesters DBC1 away from the nucleus, thereby removing a brake on DBC1 nuclear targets, such as SIRT1. This study provides new mechanistic insights into the DBC1–MCC axis as a new APC independent β-cat inhibitory pathway.**



The mutated in colorectal cancer (MCC) gene was initially discovered because of its proximity to the *APC* gene on chromosome 5,[1] but its importance as a tumor suppressor gene has only recently been recognized. MCC mutations can drive colon carcinogenesis as shown through an unbiased genetic screen of a mouse model of colorectal cancer.[2] MCC mutations have been found in a wide range of different cancers but in colorectal cancer the *MCC* gene is most commonly altered through methylation (40–50%), which causes epigenetic silencing of gene expression.[3,4] In liver cancer, MCC expression is lost through LINE-1 retrotransposition events[5] or microRNA targeting.[6] Furthermore, SNPs within the MCC gene and its expression levels are associated with responsiveness to chemotherapy treatment in acute myeloid leukaemia.[7] Limited data are available for other cancer types, except for lung cancer where expression of MCC is also variable but promoter methylation is rare.[8]

A number of new biological functions have been identified for MCC, including repression of beta-catenin (β-cat) signaling.[4,9–11] Hyperactivation of the β-cat pathway is widely accepted as a major event in colorectal carcinogenesis, largely due to *APC* mutation. Mutated APC loses its ability to direct cytoplasmic β-cat for degradation, resulting in nuclear accumulation and inappropriate activation of β-cat–mediated transcription. MCC also inhibits β-cat dependent transcription *in vitro*, even in the absence of APC, through a mechanism independent and distinct from APC.[4] This has led to the suggestion that inhibition of Wnt signaling is the main tumor suppressor function of MCC. This suggestion was based

**Key words:** MCC, DBC1, SIRT1, colon cancer, tumor suppressor, beta-catenin

**Abbreviations:** DBC1: deleted in breast cancer 1; MCC: mutated in colorectal cancer

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**What's new?**
**Mutations or silencing of a gene called MCC can promote colon carcinogenesis in vivo. In this study, the authors found that the MCC protein can repress beta-catenin (β-cat ) signaling, by sequestering a protein called DBC1 away from the nucleus. Overexpression of β-cat is associated with many cancers. The study also found that repression of β-cat by the MCC/DBC1 complex is strongly impaired when a disease-associated mutation in MCC is present. This further strengthens the role of MCC as a bona fide tumor suppressor.**



on our finding that *MCC* methylation is more frequent in serrated polyps (80%) where *APC* mutations are less common but subsets of serrated polyps still display β-cat activity deregulation.[3,12] Remarkably, a recent study also showed that loss of MCC expression in liver tissue led to oncogenic β-cat signaling, which is expected to promote carcinogenesis in the context of HBV-induced liver inflammation.[5] Taken together, the overwhelming evidence of the oncogenic property of β-cat hyperactivation and the newly emerging 'driver' carcinogenic role of the *MCC* defect justifies a thorough analysis of how loss of *MCC* expression affects β-cat signaling.

To further characterize the mechanism by which MCC contributes to β-cat transcriptional repression, we used a yeast two-hybrid approach and identified deleted in breast cancer 1 (DBC1, also known as KIAA1967) as a new MCC-interacting partner. DBC1 is an important regulator of a wide variety of cellular processes including gene expression, chromatin remodeling, cell proliferation, apoptosis and metabolism. DBC1 inhibits its two deacetylase proteins SIRT1 and HDAC3 and a histone methyl transferase SUV39H1.[13–16] In addition, DBC1 is also known to regulate a number of transcription factors including BRCA1,[17] androgen receptor[18] and ERalpha/beta.[19,20] Several recent lines of evidence are pointing towards a role of the DBC1 pathway as a positive regulator of β-cat activity through its inhibition of SIRT1.[21–23] However, despite the predicted role of DBC1 as an activator of the β-cat pathway, its direct involvement or the mechanism has not been determined yet. Also, the details of how DBC1 itself is regulated are poorly understood.

In this study, we show that MCC represses β-cat transcriptional activity by sequestering DBC1 in the cytoplasm. Importantly, this repression is inhibited by the presence of a disease-associated mutation, supporting this function as a *bona fide* MCC tumor suppressor activity.

## Material and Methods

### Cell culture

RKO, 293T, HCT116 and SW620 cell lines were propagated as recommended by the American Type Culture Collection. All cancer cell lines were obtained from the American Type Culture Collection and authenticated by Cell Bank Australia by short tandem repeat (STR) profiling, in line with the draft international standard currently being prepared by the ATCC Standards Development Organisation Workgroup ASN-0002 (04/05/2011).

### Antibodies

The following antibodies were used: anti-MCC (BD Bioscience #610740), B-cat (BD Bioscience #610153), B-cat-K49ac (Cell Signaling #9534S), anti-DBC1 (Sigma HPA019943), GAPDH (4300, Ambion, Austin, TX), Horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Amersham, GE Healthcare).

*Yeast two-hybrid screens.* Yeast two-hybrid screens were performed as previously described.[24] MCC full length (p23508) was used as a bait and a cDNA library from a human colon adenocarcinoma epithelial cell line was used as a prey [IMAGE Library (NIH_MGC_15), ID = 1,428]. Interactions were identified by the ability of the yeast to grow on media lacking uracil and positive clones were sequenced.

*Knock-down, transfection and site directed mutagenesis.* MCC siRNA were used as previously described.[9] DBC1 siRNA duplexes were 21 bp as follows: DBC1 siRNA sense strand, 5′-AAACGGAGCCUACUGAACAUU-3′, nontargeting siRNA (D001210-03-20; Dharmacon) was used as control. Transfection was performed twice, 24 hr apart, with 10 nM siRNA using DharmaFECT1 reagent according to the manufacturer's instructions. Cells were harvested or fixed 72 hr after the first transfection. The empty pQCXIP vector, pQCXIP containing full-length MCC cDNA (amplified from clone MGC:12731, Invitrogen) or MCC mutants were transfected using Lipofectamine2000 (Invitrogen, Carlsbad, CA). Site directed mutagenesis was carried out as previously described.[25]

### β-cat TCF/LEF luciferase assay

Exponentially growing cells in six-well plates were transfected with either the β-cat/TCF/LEF-(TOF) luciferase reporter construct alone, β-cat/TCF/LEF-(FOP) luciferase reporter construct alone or co-transfected with the indicated MCC plasmid using Xtreme Gene transfection reagent. Luciferase reporter plasmids M50 Super 8× TOPFlash and M51 Super 8× FOPFlash were gifts from Randall Moon, University of Washington.[26] The beta-galactosidase construct was co-transfected as a normalization control. After an overnight incubation, the cells were stimulated with either control or Wnt3a-conditioned medium, harvested and lysed in Tropix Galacto-Star lysis solution (Applied Biosystems, T2071). Ten microliters of each lysate was incubated with luciferase Assay Reagent (Promega E1505) and luciferase reporter assays were performed. The β-cat/TCF/LEF luciferase activity was normalized with the value of the corresponding beta-galactosidase dependent luciferase activity (Applied

Biosystems T2254, T2239) and the ratio ($n = 3$, mean ± S.D.) was statistically analyzed (*t*-test).

### Immunofluorescence microscopy

RKO cells were cultured as described above on glass coverslips and processed as previously described.[27] Optical sections were analyzed by confocal microscopy on an inverted microscope (DM IRE2, Lecia) using a 63×/1.4 NA HC Plan 10×/25 objective lens (Leica). The contrast was adjusted for all images with the same settings.

### Co-immunoprecipitation, cell fractionation and western blotting

Immunoprecipitation was performed on whole cell lysate resuspended in cold TNE buffer using the appropriate antibody at 4°C O/N. Immunoprecipitates were resolved on 10% or 8% SDS-PAGE gels. Proteins were transferred to nitrocellulose (Millipore, Bedford, MA), immunoprobed and detected by enhanced chemiluminescence (Pierce Chemical, Rockford, IL). Cellular fractionation was conducted by lysing cells in Nuclear Fractionation Buffer (HEPES 20 mM pH 7.2, KCl 10 mM, $MgCl_2$ 2 mM, NP-40 0.5%, protease inhibitor) and homogenized using a dounce homogenizer. Homogenate was centrifuged to sediment nuclei and the supernatant removed and re-centrifuged to pellet cell debris. The resultant supernatant comprises the non-nuclear fraction. The nuclear pellet was washed and resuspended in Nuclear Fractionation Buffer which is supplemented with 0.5 M NaCl to lyse nuclei. This was again centrifuged and the supernatant obtained comprised the nuclear fraction. Nicotinamide (Sigma-Aldrich Chemical, N3376) was prepared as a 1 M stock solution with phosphate-buffer saline (PBS).

## Results

### The ability of MCC to repress β-cat transcription is strongly impaired by the presence of a disease-associated patient mutation

We first analyzed the effect of MCC overexpression or knockdown on β-cat signaling using the TCF/LEF Top-flash luciferase assay in colorectal cell lines.[4] RKO cells (wild type for β-cat and APC) were transiently co-transfected with MCC or vector alone and the β-cat/TCF/LEF reporter plasmid for 36 hr. Expression of MCC in this cell line resulted in a robust inhibition of β-cat transcriptional activity (Fig. 1*a*). To further confirm the involvement of MCC in β-cat-mediated transcription, MCC was targeted by RNA interference (siRNA) in HCT116 cells, which express endogenous MCC. To reveal possible off-target effects, we used two different siRNA sequences that target different regions of MCC mRNA. Consistent with a role of MCC as a repressor of β-cat activity, MCC depletion resulted in an increase in β-cat activity (Fig. 1*b*). However, MCC over-expression or knock-down had no effect on the transcriptional activity of Fop-Flash vector carrying a mutant TCF binding site (data not shown).

Strikingly, following MCC expression in RKO cells a robust dose-dependent reduction of β-cat transcriptional activity was observed both in low basal (Fig. 1*c*) as well as in high Wnt3a stimulated conditions promoter activity (Fig. 1*d*). Western blot analysis showed that MCC-mediated repression was not associated with a reduction in the total amount of β-cat suggesting an alternative mode of regulation compared to APC, which mediates its transcriptional repression by promoting β-cat degradation (Figs. 1*a* and 1*b*). Taken together, MCC regulates the Wnt pathway in cells with wild type APC/β-cat (RKO), mutated β-cat (HCT116) or mutated APC (SW480[4]). These data suggest a novel mode of regulation independent of the status of β-cat pathway activation.

Silencing of MCC expression is a common defect in colorectal cancer[3,4] but disease-associated missense mutations have also been described.[1] We tested the tumor-associated mutation (R506Q) and showed that this single amino acid substitution failed to repress β-cat transcriptional activity, compared to MCC-WT or MCC bearing a single nucleotide polymorphism P486L (Fig. 1*e*). Interestingly, MCC bearing R506Q missense mutation was previously shown to prevent the MCC-induced G1-S phase cell cycle arrest.[28] These data highlight a possible link between β-cat repression by MCC and its anti-proliferative role. More importantly these data strongly suggest that one of the tumor suppressor functions of MCC is to repress β-cat transcriptional activity.

### Identification of DBC1 as a MCC interacting partner

To further characterize the mechanism by which MCC contributes to transcriptional repression of β-cat, we used a yeast two-hybrid approach to identify MCC interacting partner(s). MCC full length clone (AN:P23508) was used as bait and a cDNA library from a human colon adenocarcinoma cell line was used as prey (IMAGE Library cDNA; NIH_MGC_15, ID = 1,428). Interactions were identified by the ability of yeast to grow on media lacking uracil and positive clones were sequenced. Three independent clones were identified as DBC1, Deleted in Breast Cancer1 (BC018269.1) (clone-1: aa190-end, clone-2: aa189-end, clone-3: aa207-end). Each positive clone was re-cloned and individually re-tested by yeast two-hybrid (Fig. 2*a*). MCC/DBC1 interaction was confirmed by pull-down assay, using transiently expressed HA-DBC1 and Flag-MCC in 293T epithelial cells. HA-DBC1 successfully immunoprecipitated MCC (Fig. 2*b*). Reciprocally, FLAG-MCC successfully immunoprecipitated HA-DBC1 (Fig. 2*c*). We next showed that MCC/DBC1 interaction occurs endogenously by immunoprecipitating endogenous DBC1 from SW620 epithelial colorectal cell line and blotting for endogenous MCC (Fig. 2*d*).

We next tested the impact of MCC-R506Q mutation, shown to affect MCC ability to repress β-cat transcriptional activity, and found that the presence of this mutation reduced the ability of MCC to bind DBC1 compared to MCC-WT (Fig. 2*e*). This suggests that MCC–DBC1 interaction is important for MCC-induced β-cat transcriptional repression.

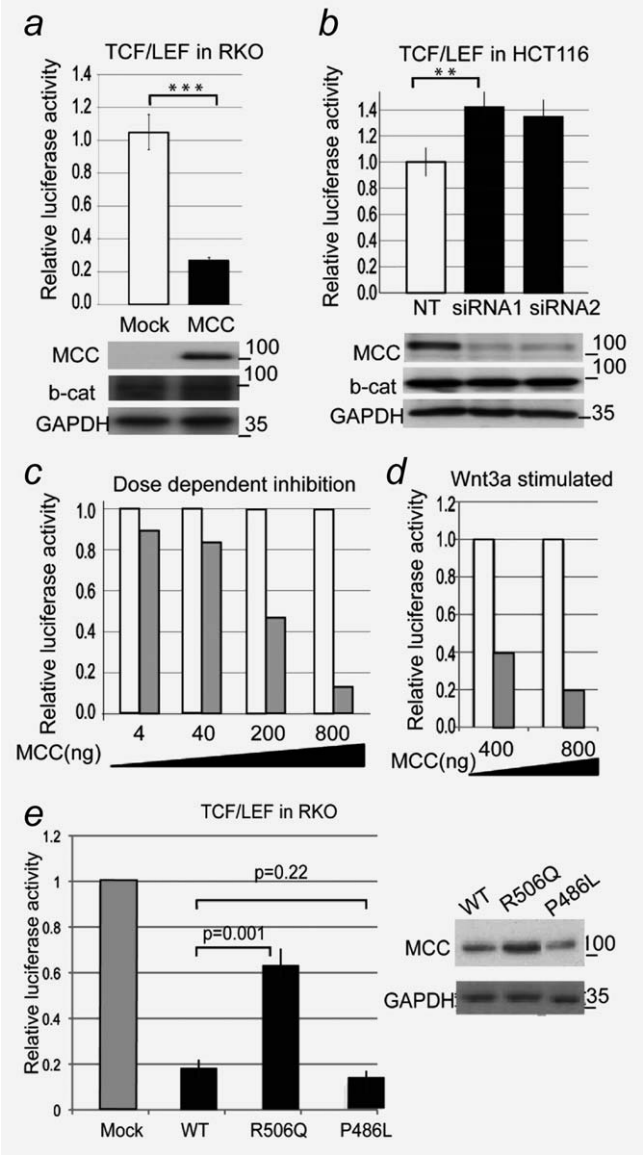


**Figure 1.** MCC ability to repress β-cat transcriptional activity is strongly impaired by the presence of a disease-associated patient mutation. *a*: RKO cells were transfected with either 200 ng of MCC or pQCXIP control plasmid together with 1 μg of the β-cat/TCF/LEF. The *y*-axis shows normalized relative luciferase activity, data are represented as mean ± S.D., $n = 3$. *b*: HCT116 cells were transfected with two independent MCC siRNA or non-targeted siRNA (NT) for 36 hr prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean ± S.D., $n = 3$. Lysates not used for luciferase assay were pooled and analyzed by Western blotting for MCC, β-cat and GAPDH. *c*: RKO cells were co-transfected with 4, 40, 200 or 800 ng of MCC (grey bars) or equivalent amount of pQCXIP control plasmid (white bars) together with 1 μg of the β-cat/TCF/LEF reporter construct. The *y*-axis shows relative normalized luciferase activity, data are represented as mean of two independent experiments. *d*: RKO cells were co-transfected with 400 or 800 ng of MCC (grey bars) or pQCXIP control plasmid (white bars) together with 1 μg of the β-cat/TCF/LEF reporter construct. Cells were stimulated for 16 hr with Wnt3A-medium prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean of two independent experiments. *e*: RKO cells were co-transfected with 200 ng of MCC WT, MCC mutant or 200 ng of pQCXIP control plasmid together with 1 μg of the β-cat/TCF/LEF reporter construct. Cells were stimulated for 16 hr with Wnt3A-medium prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean ± S.D., $n = 3$. Lysates not used for luciferase assay were pooled and analyzed by Western blotting.

### DBC1 depletion strongly represses β-cat transcriptional activity and prevents further MCC mediated β-cat transcriptional repression

Despite the predicted role of DBC1 as a positive regulator of the β-cat pathway through SIRT1 inhibition,[21–23] its direct involvement has not yet been shown. We first tested the effect of DBC1 knockdown on β-cat transcriptional activity in RKO cells. DBC1 depletion resulted in robust β-cat transcriptional repression, similar to the one observed following MCC expression (Fig. 3*a*). Furthermore, DBC1 depletion together with Fop-Flash vector

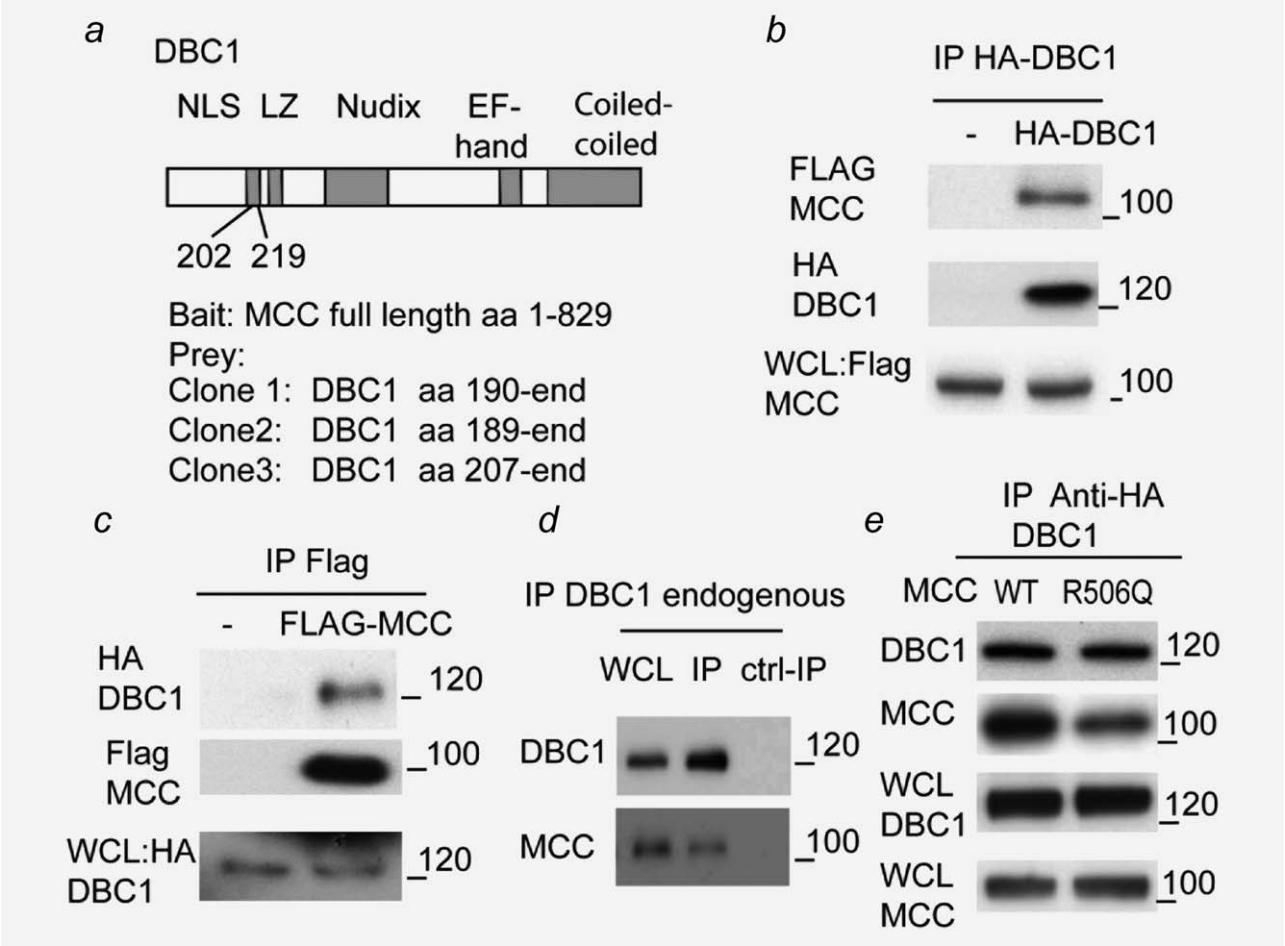


**Figure 2.** MCC interacts with DBC1. *a*: MCC full length (p23508) was used as a bait and a cDNA library from a human colon adenocarcinoma epithelial cell line was used as a prey. Interactions were identified by the ability of the yeast to grow on media lacking uracil and positive clones were sequenced. Three clones were identified as DBC1. Shown is a schematic diagram of full-length DBC1 (*FL*): *NLS*, nuclear localization signal; LZ, Leucine Zipper. *b*: 293T cells were co-transfected with expression plasmids for HA-DBC1 and FLAG-MCC or vector control. DBC1 was immunoprecipitated (*IP*) with anti-HA antibody. Immunoprecipitates were run and blotted for MCC and HA-DBC1. *wcl*, whole cell lysate. *c*: 293T cells were co-transfected with expression plasmids for HA-DBC1 and FLAG-MCC or vector control. MCC was immunoprecipitated (*IP*) with anti-FLAG antibody and immunoprecipitates blotted for DBC1 (anti-HA) and MCC (anti-Flag). *wcl*, whole cell lysate. *d*: Endogenous DBC1 was immunoprecipitated from SW620 cell lysate. Immunoprecipitates were run and blotted for DBC1 and MCC. *e*: MCC-WT or MCC-R506Q were co-transfected with HA-DBC1. Twenty-four hours post-transfection, lysates were harvested and DBC1 was immunoprecipitated using HA antibody. Immunoprecipitates were run and blotted for MCC and DBC1.

carrying a mutant TCF binding site had no effect on the transcriptional activity. To address the role of DBC1 on MCC mediated β-cat repression, we repeated the TCF/LEF assay in RKO cells following DBC1 knockdown with concurrent ectopic expression of MCC. Following DBC1 depletion, MCC expression failed to further repress β-cat transcription (Fig. 3*b*).

Taken together, these data support the predicted role of DBC1 as a positive regulator of β-cat transcriptional activity. As there was no synergistic effect between DBC1 depletion and MCC expression, these two modifications may target the same pathway. This raises the possibility that MCC may inhibit β-cat directed transcription through its interaction with DBC1. We next proceeded to define the regions where the two proteins interact and whether the interaction affects MCC or DBC1 subcellular localization.

### MCC interacts outside the classical DBC1 leucine zipper binding site

As DBC1 inhibits the activity of several proteins through an interaction with its N-terminal Leucine Zipper, we first hypothesized that MCC could physically compete with other target proteins for their binding with DBC1. DBC1 contains relatively well-defined functional domains (Fig. 4*a*). In order to narrow down the region(s) of DBC1 required for the interaction with MCC, we repeated the immunoprecipitation assays using DBC1 deletion constructs. This analysis showed that deletion of the N-terminal region of DBC1, including the nuclear localization signal and the Leucine Zipper, did not abrogate the interaction with MCC. Furthermore, deletion of the C-terminal coiled-coil and EF-hand domains had no effect on the interaction (Fig. 4*b*). This finding was

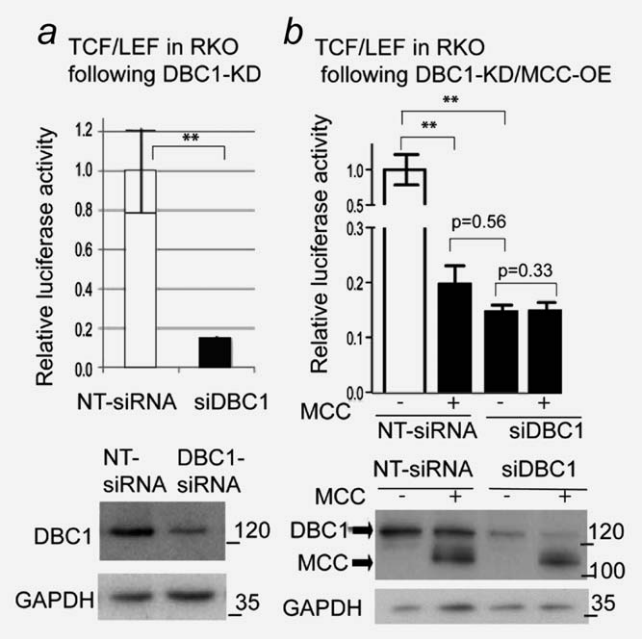


**Figure 3.** DBC1 depletion strongly represses β-cat TA and prevents MCC-mediated β-cat repression. *a*: RKO cells were transfected with DBC1 siRNA or non-targeted siRNA with 1 μg of the β-cat/TCF/LEF reporter construct. Cells were stimulated for 16 hr with Wnt3A-medium prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean ± S.D., $n = 3$. *b*: RKO cells were transfected with DBC1 siRNA or non-targeted siRNA and expression plasmids for MCC or vector control with 1 μg of the β-cat/TCF/LEF reporter construct. Cells were stimulated for 16 hr with Wnt3A-medium prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean ± S.D., $n = 3$. Lysates not used for luciferase assay were pooled and analyzed by Western blotting for MCC, DBC1 and GAPDH.

surprising as all known DBC1 interacting partners have been shown to interact *via* the DBC1 Leucine Zipper domain (residues 243–264). Taken together, and consistent with the yeast two-hybrid data, our data narrowed down the interaction with MCC to the central region of DBC1 (264–704). A conserved NUDIX domain (aa339–462) has been identified in this region[29] (Fig. 4*c*), but this domain was not required for MCC–DBC1 interaction (Fig. 4*d*).

We further subdivided the interacting region into three domains; a N-terminal region (aa559–610), a central glutamic-acid rich region (aa610–670) and a highly phosphorylated C-terminal part (aa670–704). We repeated the co-immunoprecipitation assays using three deletion constructs and identified DBC1 glutamic acid rich region (aa610–670) as being required for MCC interaction (Fig. 4*e*). Conservation analyses of this region suggest a selective pressure for the maintenance of acidic residues, suggesting a potential role for salt bridges in MCC/DBC1 interaction. Hence, glutamic acid residues are either conserved among species or substituted by aspartic acid, another negatively charged residue. A similar observation can be made for aspartic acid residue substitution by glutamic acid (Fig. 4*f*).

### MCC nuclear localization is not required for MCC/DBC1 interaction

It has been suggested that MCC nuclear localization might be required for its β-cat repressive role.[4] Although a conserved bi-partite NLS domain was predicted at the C-terminal of MCC,[4] its functional relevance on MCC sub-cellular localization or β-cat transcriptional activity has not been tested (Fig. 5*a*). To address this question, we deleted the MCC bi-partite NLS domain (Delta-766–782) and assessed MCC cellular localization. Cellular fractionation showed that MCC-WT is present in the cytoplasm and the nucleus while MCC-ΔNLS was detected only in the cytoplasm (Fig. 5*b*). We next tested the ability of MCC-ΔNLS to inhibit β-cat transcriptional activity and found that MCC-ΔNLS repressed β-cat as efficiently as MCC-WT (Fig. 5*c*). Taken together, our data show that the MCC bi-partite NLS domain is functional. However, nuclear localization of MCC is not necessary for β-cat transcriptional repression. In addition, immunoprecipitation experiments showed that MCC deleted of its NLS can still bind to DBC1 (Fig. 5*d*).

Another region of interest in the MCC protein is the region between residues 633 and 650, where post-translational modifications have been described, such as acetylation at Lysine-633, Lysine-641, Lysine-643 and Lysine 644(referred as Acetyl-rich region or Ac-R) (Fig. 5*a*). Lysine acetylation is considered as the main modification regulating transcriptional activity, in particular through the modification of nuclear transcription regulators and histones.[30] However, despite our prediction, deletion of MCC acetyl-rich region did not affect the ability of MCC to repress β-cat transcriptional activity or alter its subcellular localization (Figs. 5*b* and 5*c*).

### Expression of MCC wild type, but not MCC bearing R506Q mutation, induces DBC1 cytoplasmic relocalization and β-catenin de-acetylation

As our experiments with the MCC-ΔNLS mutant indicated that MCC could repress β-cat transcriptional activity in the cytoplasm, we next tested whether MCC could regulate DBC1 sub-cellular localization. GFP-MCC and HA-DBC1 were expressed in RKO cells for 24 hr before being fixed and stained for DBC1. As expected, DBC1 was primarily observed in the nucleus of mock-transfected cells (Fig. 6*a*, red arrows). However, there was a pronounced DBC1 cytoplasmic relocalization in cells co-transfected with GFP-MCC in RKO cells (Fig. 6*a*, white arrows). Quantification of the number of RKO cells showing DBC1 nuclear, nuclear/cytoplasmic or cytoplasmic localization in the presence or absence of MCC expression is shown in Figure 6*b*. As MCC bearing R506Q mutation strongly impair MCC ability to repress β-cat transcriptional activation, we next looked at the subcellular localization of MCC mutant R506Q and its ability to relocate DBC1 to the cytoplasm compared to wild type (Fig. 6*a*, bottom panel). The number of cells showing DBC1 cytoplasmic

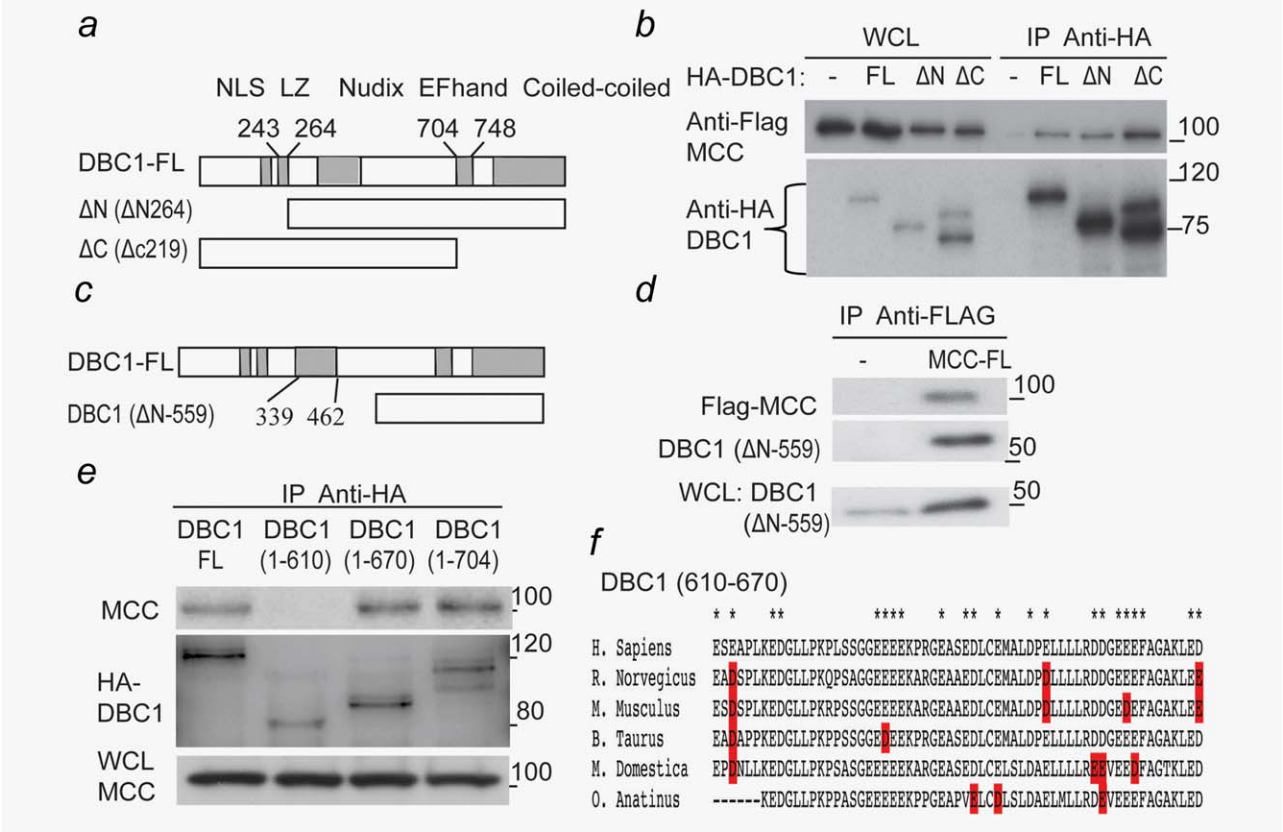


**Figure 4.** MCC does not interact through the classical DBC1 Leucine Zipper binding domain. *a*: Shown is a schematic diagram of DBC1 deletion constructs used for our pull down assays. Full-length DBC1 (FL), DBC1 N-terminal deletion (ΔN264), DBC1 C-terminal deletion (ΔC219). *b*: Plasmids encoding HA-tagged full-length DBC1 (*FL*), deletion mutants, or vector control (-) were transfected in 293T cells along with FLAG-MCC. Cell lysates were immunoprecipitated with anti-HA antibody. Immunoprecipitates were run and blotted for MCC (anti-FLAG) and DBC1 (anti-HA). *wcl*, whole cell lysate. As previously described, we also observed that the DBC1 (ΔC219) migrates slower than expected and appears as doublets.[15] *c*: Schematic diagram of DBC1 deletion. *d*: DBC1 N-terminal deletion (ΔN559) that lacks DBC1 Nudix domain (339–462) was co-transfected with MCC-FL or vector control. Lysates were immunoprecipitated using Flag antibody. Immunoprecipitates were run and blotted for MCC and DBC1. *e*: DBC1 deletion mutants were co-transfected with MCC. Twenty-four hours post-transfection, lysates were harvested and DBC1 was immunoprecipitated using HA antibody. Immunoprecipitates were run and blotted for MCC and DBC1. *f*: Multiple alignments show the degree of conservation of this domain. Acidic residues are marked with a star and conserved acidic residues are highlighted in red. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

relocalization following MCC-R506Q expression was strongly reduced compared to MCC wild type (Fig. 6*b*).

SIRT1, the main DBC1 target, deacetylates β-cat-K49 and suppresses the ability of β-cat to activate transcription and drive cell proliferation.[21,31] We next looked at the effect of MCC re-expression on β-cat-K49 acetylation. In line with loss of SIRT1 repression, expression of wild type MCC but not MCC-R506Q mutant resulted in β-cat-K49 deacetylation (Fig. 6*c*). We tested the effect of MCC expression in the presence of Nicotinamide, a SIRT1 inhibitor. In agreement with a role of SIRT1, we found that Nicotinamide reversed MCC-induced β-cat-K49 deacetylation (Fig. 6*d*). Thus, together with the luciferase reporter assays (Figs. 1 and 3) these data suggest that the cytoplasmic MCC–DBC1 interaction sequesters DBC1 away from the nucleus, thereby removing a brake on nuclear DBC1 targets SIRT1 and allowing it to deacetylate β-cat.

## Discussion

There is growing evidence to suggest that the MCC gene is a potent tumor suppressor in mouse and human tissue and that MCC represses the β-cat pathway.[2,4,5,32] Data from us and others[4] show that the inhibitory activity of MCC is independent of APC or β-cat status as it is observed in RKO (wild type APC/β-cat), HCT116 cells (β-cat mutant) and SW480 (APC mutant); it is dose dependent, that is, increases with the amount of MCC expressed and is also evident in basal and Wnt3a stimulated conditions (Figs. 1*c* and 1*d*). However, MCC inhibitory activity is not associated with a change in the total amount of β-cat, suggesting an alternative mode of regulation compared to APC (Figs. 1*a* and 1*b*). Importantly, here we show that MCC bearing a disease-associated mutation fails to fully repress β-cat activity supporting the view that MCC mediated β-cat repression is a bona-fide tumor suppressor function (Fig. 1*e*).

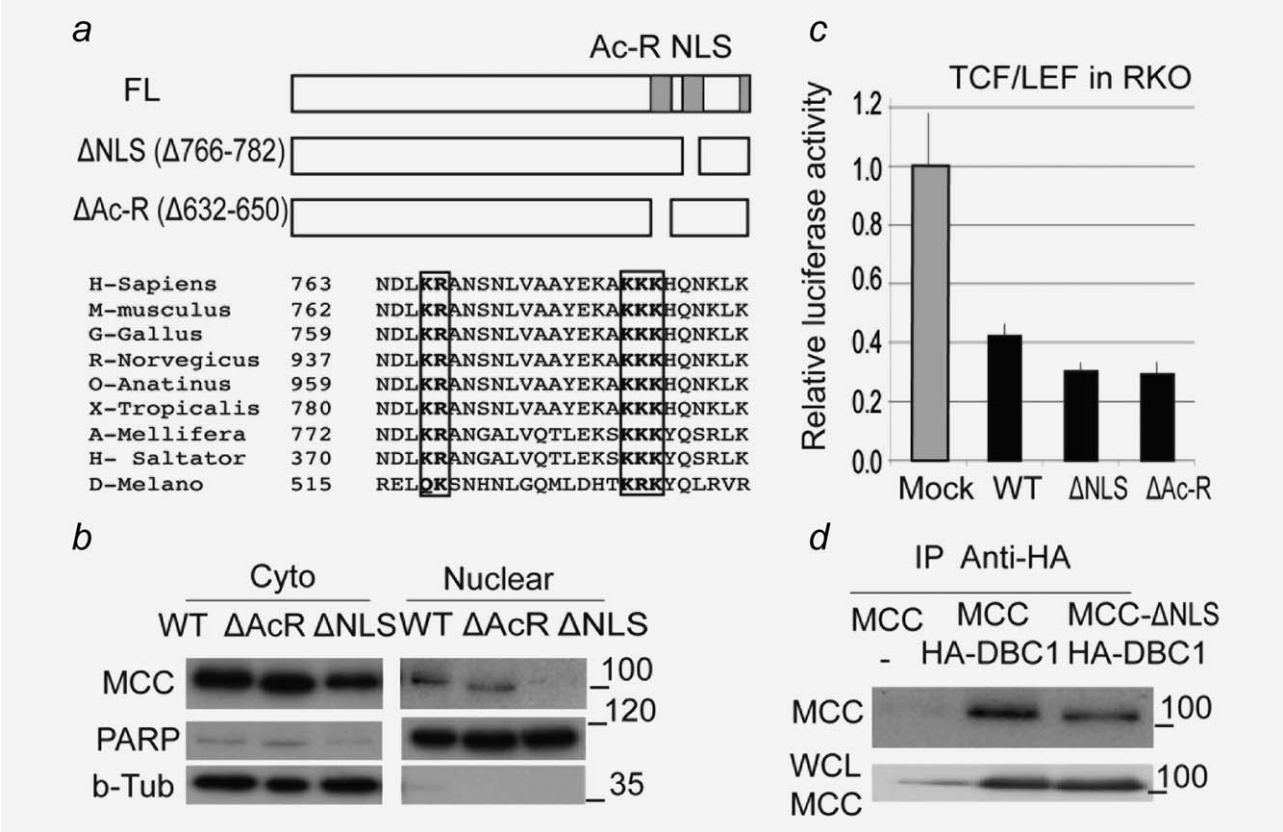


**Figure 5.** MCC nuclear localization is not required for MCC/DBC1 interaction. *a*: Schematic representation of MCC full length (FL), MCC deleted of its bi-partite nuclear localization signal (ΔNLS) or MCC deleted of its lysine-acetyl rich region (ΔAc-R). Multiple alignments are showing the high degree of conservation of the bi-partite NLS. *b*: MCC full length, MCC(ΔNLS) or MCC(ΔAc-R) were transfected in 293T for 24 hr before proceeding to cytoplasmic/nuclear fractionation. The different fractions were run and blotted for MCC, b-Tubulin (cytoplasmic marker) and PARP (nuclear marker). *c*: RKO cells were transfected with 200 ng of MCC full length, MCC(ΔNLS) or MCC(ΔAc-R) or 200 ng of pQCXIP control plasmid together with 1 μg of the β-cat/TCF/LEF reporter construct. Cells were stimulated for 16 hr with Wnt3A-medium prior to luciferase reporter assay. The *y*-axis shows relative normalized luciferase activity, data are represented as mean ± S.D., $n = 3$. *d*: Plasmids encoding full-length MCC or MCC deleted of its NLS were transfected along with HA-DBC1 in 293T cells. Cell lysates were immunoprecipitated for DBC1 (anti-HA). Immunoprecipitates were run and immunoblotted for MCC.

We have identified DBC1 as a novel interacting partner of MCC and show new evidence that DBC1 is a regulator of β-cat transcriptional activation. We found that MCC binds DBC1 *via* a newly identified domain, outside the Leucine Zipper region, and that this binding results in DBC1 cytoplasmic relocalization, which is expected to impair the ability of DBC1 to inhibit its targets in the nucleus. In line with this model, transient MCC expression resulted in β-cat-K49 deacetylation, which is also a well-described SIRT1 function.[21,31] Importantly, MCC bearing the patient derived mutation R506Q, which failed to repress β-cat transcriptional activity, also failed to deacetylate β-cat and failed to adequately bind and relocate DBC1 to the cytoplasm. Thus these data support the sequestration of DBC1 in the cytoplasm as the likely mechanism of MCC-mediated β-cat repression. It is worth noting that MCC has also been shown to repress cell proliferation in a number of cell line models[4,9] and MCC bearing the disease-associated mutation R506Q failed to block cell proliferation.[28] Taken together, these data provide a possible link between the MCC/β-cat repression and MCC anti-proliferative properties.

Whether DBC1 is a tumor suppressor or promoter remains unclear and it is possible that this role varies between different types of cancer. The DBC1 gene was initially cloned from a chromosomal region (8p21) homozygously deleted in breast cancers but DBC1 overexpression is associated with a poor prognosis in colon cancer.[33] DBC1 is involved in a wide variety of cellular processes and one of its key functions is inhibition of the deacetylase SIRT1.[13] We propose a mechanism by which MCC-mediated DBC1 cytoplasmic relocalization impairs the ability of DBC1 to inhibit SIRT1, providing a potential new mode of DBC1 regulation. Several studies are pointing towards a role of SIRT1 as a tumor suppressor in colorectal cancer and importantly this function could be mediated

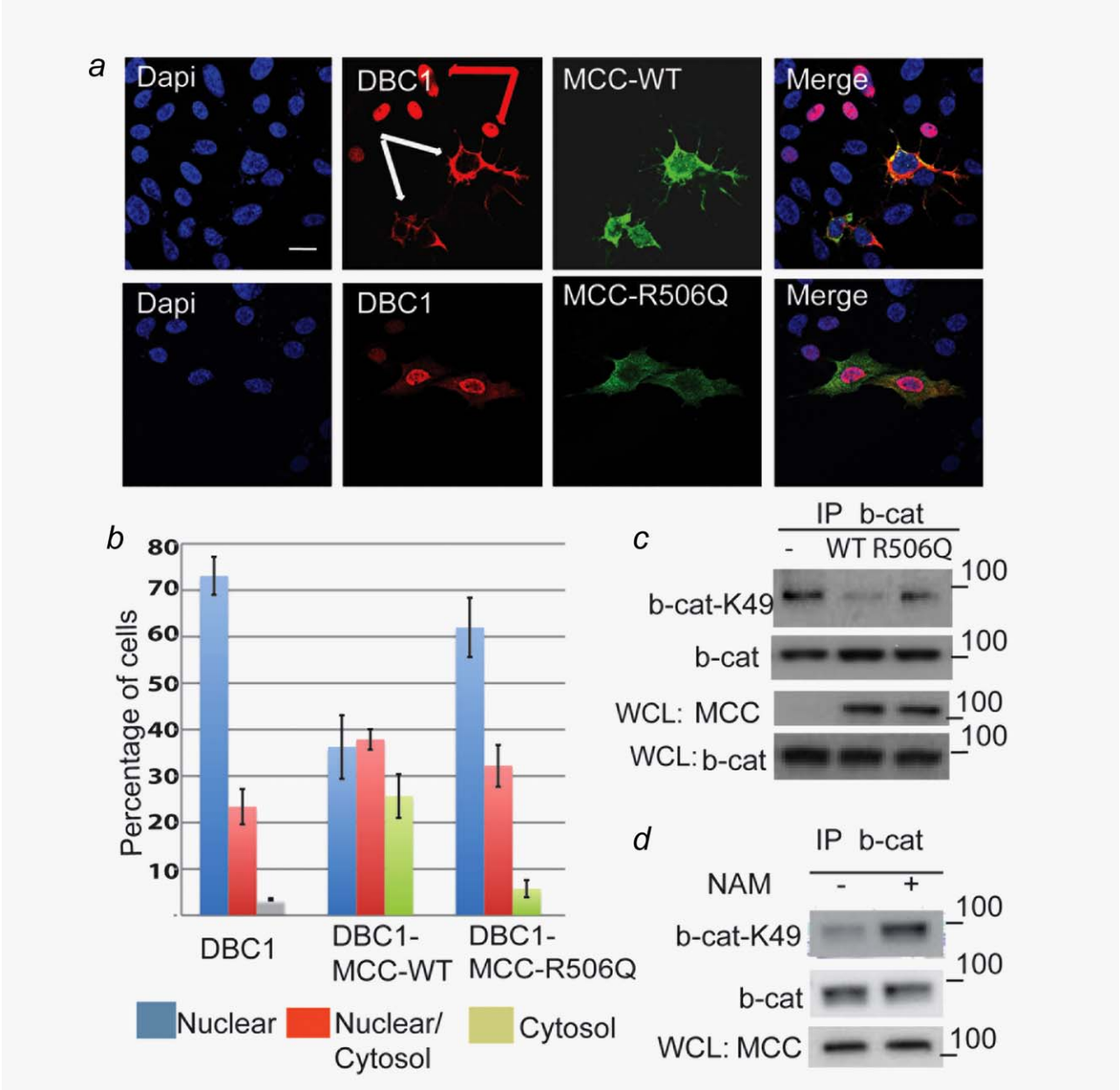


**Figure 6.** Expression of MCC wild type, but not MCC bearing R506Q patient mutation, induces DBC1 cytoplasmic relocalization and β-cat de-acetylation. *a*: RKO cells were transfected with HA-DBC1, GFP-MCC-wild type, GFP-MCC-R506Q or vector control. Twenty-four hours post-transfection, the cells were fixed and stained for DBC1 (anti-HA). Shown is a representative panel of cells transfected with HA-DBC1 only (red) or co-transfected with GFP-MCC. The nucleus was stained using Dapi. *b*: Quantification of the number of cells co-transfected with MCC-WT or MC-R506Q showing DBC1 nuclear, cytoplasmic/nuclear or cytoplamic localization. Graph values represent the mean score of at least 200 cells from two independent experiments and deviation from the mean. *c*: MCC-WT, MCC-R506Q or vector control was co-transfected with β-cat for 24 hr in 293T cells. Cell lysates were immunoprecipitated for β-cat. Immunnoprecipitates were run and immuno-blotted for β-cat-K49 and total β-cat. *d*: MCC-WT was co-transfected with β-cat for 24 hr in 293T cells in the presence of Nicotinamide (10 mM) or vehicle only (PBS). Cell lysates were immunoprecipitated for β-cat. Immunnoprecipitates were run and immunoblotted for β-catenin-K49 and total β-catenin.

through inhibiting β-cat activity.[21–23] In agreement with this model, SIRT1 also inhibits proliferation of pancreatic cancer cells by suppressing β-cat transcriptional activity[22] as well as in hepatic cells.[23] DBC1 also has SIRT1 independent functions and it is intriguing that both MCC and DBC1 are involved in the regulation of cellular responses to UV-induced DNA damage.[9,34] Thus, it remains to be established whether the MCC/DBC1 interaction described here is also important in regulating the DNA damage response.

In conclusion, this work describes a new mode of regulation of the β-cat pathway independent of the classical APC/β-cat degradation pathway. A recent study found that loss of MCC expression led to oncogenic β-cat/Wnt signaling in human liver tissue, which is expected to promote carcinogenesis in the context of HBV-induced liver inflammation.[4] This study highlights the likely broader impact of the tumor suppressor role of MCC in cancer. Taken together, our findings could have major implications in the management of MCC deficient tumors as well as lead to new therapeutic avenues to target oncogenic β-cat activity.

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