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Deregulation of CRAD-Controlled Cytoskeleton Initiates Mucinous Colorectal Cancer via β -Catenin

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Abstract

The epithelial integrity is maintained by the cytoskeleton and cell adhesion. However, it remains unknown how deregulated cytoskeleton is associated with cancer. We identified *Cancer-related Regulator of Actin Dynamics (CRAD)* as frequently mutated or transcriptionally downregulated in colorectal cancer (CRC). We found that CRAD stabilizes the cadherin-catenin-actin (CCA) complex *via* capping protein inhibition. CRAD loss inhibits F-actin polymerization and subsequently disrupts the CCA complex, which leads to β -catenin release and Wnt signaling hyperactivation. In mice, *CRAD* knockout induces the epithelial cell integrity loss and Wnt signaling activation, resulting in intestinal mucinous adenoma development. With *APC* mutation, *CRAD* knockout initiates and accelerates mucinous and invasive adenoma development in the colorectum. These results define CRAD as a tumor suppressor, of which inactivation deregulates the cytoskeleton and hyperactivates Wnt signaling, initiating mucinous CRC. Our study reveals the unexpected roles of an actin cytoskeletal regulator in maintaining epithelial cell integrity and suppressing tumorigenesis.

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Author contributions

Y.-S.J. and J.-I.P. conceived the experiments. Y.-S.J., W.W., S.J., J.Z., M.S., M.J.K., E.M.L., J.S., and J.-I.P. performed the experiments. Y.-S.J., J.C., P.D.M., S.Z., and J.-I.P. analyzed the data. Y.-S.J. and J.-I.P. wrote the manuscript.

Disclosure of Potential Conflicts of Interest

The authors declare no competing financial interests.

Epithelial monolayer integrity is maintained to significant extents *via* cell-cell adhesion, the cytoskeleton, and basement membrane interaction¹⁻³. Cell adhesion proteins and the cytoskeleton are intimately associated, with prime examples being the E-cadherin-catenin complex and filamentous actin (F-actin). E-cadherin participates in cell adhesion and contact inhibition, being part of a large complex composed of catenins and additional proteins (e.g., α -, β -, and p120-catenin, vinculin, α -actinin, and eplin) that is stabilized by interactions with F-actin²⁻⁵. F-actin polymerization, which involves the addition of ATP-globular actin (G-actin) at the barbed (+) end of filaments, is controlled by capping proteins (CPs) and CP regulators⁶. CPs directly bind to and block the barbed (+) end of filaments or to ATP-G-actin, resulting in inhibition of actin assembly. Several CP regulators control CPs. For example, Formins and ENA/VASP compete with CPs for actin binding^{7, 8}. Alternatively, V-1 and phospholipids, bind to CPs and prevent CPs interactions with actin^{9, 10}. Given that F-actin stabilizes the E-cadherin-catenin complex for the maintenance of epithelial cell integrity, we hypothesized that reduced epithelial cell integrity through deregulation of the cytoskeleton and the E-cadherin-catenin complex contributes to tumorigenesis. Our unbiased and comprehensive approaches identified *CRAD* (*Cancer-related Regulator of Actin Dynamics*; *KIAA1211*, hereafter referred as *CRAD*) as a tumor suppressor in CRC.

CRAD is markedly mutated in small cell lung cancer (SCLC) patient samples, having a ranking of third following *TP53* and *RBI1*¹¹. Herein, our comprehensive approaches reveal that *CRAD* inactivation initiates mucinous intestinal tumorigenesis by disrupting the epithelial cell integrity.

CRAD inactivation in CRC

To identify potential tumor suppressor genes specifically inactivated in CRC, we selected genes in which expression is significantly downregulated in CRC. Analysis of Oncomine datasets showed that the transcriptional level of *CRAD/KIAA1211* was notably downregulated in CRC samples (Fig. 1a). Analysis of GEO datasets also indicated the significant downregulation of *CRAD* mRNA in CRC, compared to the adjacent normal samples (Fig. 1b). Immunohistochemistry (IHC) of tissue microarray (TMA) also showed decreased *CRAD* in CRC (Fig. 1c, Table S1). Furthermore, CRC cells exhibited the reduced *CRAD* mRNA expression (Fig. 1d) and protein levels (Fig. 1e), compared to intestinal epithelial cells (IECs). Additionally, *CRAD* alleles harbor nonsense mutations in CRC patient samples and CRC cell lines (Figs. 1f-1h, S1). Interestingly, in the CRC cell lines not carrying genetic mutations in *CRAD*, transcriptional downregulation of *CRAD* was observed (Fig. 1d), which was restored by inhibition of methyltransferase (Fig. S1c). These results suggest that *CRAD* is inactivated by genetic mutation or transcriptional downregulation in CRC.

Positive regulation of the actin polymerization by CRAD

Given the mutation or downregulation of *CRAD* in CRC, we hypothesized that *CRAD* is a tumor suppressor in CRC. To test this, we examined whether *CRAD* inactivation is involved in cell transformation-related cell morphological change¹², as reflected in the respective cell morphologies and actin distributions of IECs *versus* CRC cell lines (Figs. S2a, S2b). We

found that *CRAD* depletion (Figs. S2c, S2d) induced IEC shrinkage (Figs. S2e, S2f), indicated by reduced cell areas and decreased Phalloidin staining, a marker for F-actin (Fig. S2g). Conversely, *CRAD* ectopic expression increased cell area in CRC cells, with an elevated presence of the actin cytoskeleton (Figs. S2h-S2k). These results suggest that *CRAD* increases the actin cytoskeleton formation.

CRAD: an inhibitor of capping proteins

Given the enhanced actin cytoskeleton by *CRAD*, we sought to dissect its detailed molecular mechanism. Employing the tandem affinity purification and mass spectrometry, we identified *CRAD*-interacting proteins: CPs, actin/tubulin-associated proteins, and the Arp2/3 protein complex (Fig. 2a, Table S2). CPs (*CAPZA1*, *CAPZB*, and *CAPZA2*) exhibited the high scores with regards to peptide numbers. Co-immunoprecipitation (co-IPs) from cell lysates as well as pull-down assays of purified proteins validated the endogenous and direct interaction between *CRAD* and CPs, respectively (Figs. 2b, S2l). Thus, we hypothesized that *CRAD* enhances the F-actin polymerization by inhibiting CPs (Fig. 2c). We examined whether *CRAD* interferes with the binding of CPs to actin. Co-IP assays showed that ectopic *CRAD* reduced the interaction between CPs and actin (Fig. 2d, 2e). F-actin controls the cytoskeletal dynamics and stretched cell morphologies (Figs. S2e-S2k)¹³⁻¹⁵. For F-actin assembly, monomeric G-actin undergoes polymerization at the barbed (+) end through a conformational change, represented by the high ratio of F-actin to G-actin. The fractionation of F-/G-actin indicated decreased F-actin levels in *CRAD*-depleted IECs (Figs. 2f, S2m). Conversely, *CRAD* ectopic expression increased F-actin in CRC cells (Figs. S2n, S2o), consistent with the Phalloidin staining. Having determined that CPs directly bind to the barbed (+) end of F-actin and inhibit the F-actin polymerization⁶, we next asked whether *CRAD* sequesters CPs from F-actin and increases the uncapped barbed (+) ends of F-actin. Visualizing the uncapped barbed (+) ends¹⁶ showed that the ectopic expression of *CRAD* increased uncapped barbed (+) ends (Fig. 2g). Additionally, highly expressed *CRAD* increased the uncapped barbed (+) ends in the Latrunculin B (Lat B, an inhibitor of monomeric G-actin)-treated condition but not in the Cytochalasin D (Cyto D, a blocker of barbed [+]-end)-treated condition (Fig. 2g). These results suggest that *CRAD*-upregulated the actin polymerization is mainly due to the increase of the extendable barbed (+) end by inhibiting CPs, independently of the increase of monomeric G-actin. Direct CP regulators including *CARMILs*, *FAM21*, and *CD2AP* harbor a capping protein interaction (CPI) motif⁶. Interestingly, *CRAD* also contains two potential CPI motifs at 329 and 417 amino acid residues (Fig. 2h). To determine whether these predicted CPI motifs in *CRAD* are required for CP inhibition, we constructed *CRAD* mutants (M1-M4, CPI; Figs. 2i, 2j). Co-IP showed that unlike FL (full-length) and M2-M4 mutants, M1 and CPI mutants (lacking CPI motifs) did not bind to CPs (Fig. 2k), and failed to inhibit the interaction of CPs with actin (Fig. 2l). Also *in vitro* protein binding assays using the purified proteins of actin, CPs, and *CRAD*-M2 (Fig. S2p) showed that *CRAD* inhibited the actin-CP binding (Figs. 2m, S2q). Moreover, in CRC cells, M1 and CPI mutants failed to induce a stretched cell morphology (Fig. 2n) and F-actin polymerization (Fig. 2o), whereas other mutants did (M2-M4; Figs. 2n, 2o). These data suggest that *CRAD* downregulates the interaction between

CPs and the barbed (+) ends *via* the CPI motifs in CRAD, which increases F-actin polymerization (Fig. S3).

CRAD loss-activated Wnt signaling by disrupting the CCA complex

Next, we sought to determine how CRAD-modulated actin cytoskeleton is associated with intestinal tumorigenesis. Owing to deregulation of various developmental pathways in CRC, we examined the effects of CRAD on Wnt, Hedgehog, BMP, Notch, and Hippo signaling. Interestingly, CRAD overexpression downregulated Wnt/ β -catenin target genes (*AXIN2* and *CD44*; Fig. 3a). Conversely, in IECs, *CRAD* depletion augmented Wnt/ β -catenin target gene expression (Fig. 3b). Additionally, *CRAD* expression is mutually exclusive to the expression of *AXIN2* in CRC (Fig. S4a). Consistently, the level of nuclear β -catenin in IECs and CRC cell lines was inversely correlated with the expression of CRAD (Fig. S4b). These results imply that CRAD might be negatively associated with Wnt/ β -catenin signaling. Indeed, CRAD depletion increased β -catenin reporter activity, *AXIN2* expression, and the level of active β -catenin in IECs (Figs. 3c-3e). Importantly, treatment of cells with iCRT14, an inhibitor of β -catenin-TCF binding, suppressed *CRAD* depletion-induced β -catenin reporter activation (Fig. 3f). Also, Engrailed-LEF1 (Eng-LEF1), a dominant-negative mutant blocking β -catenin-mediated gene activation¹⁷, suppressed *AXIN2* upregulation in *CRAD*-depleted IECs (Fig. 3g). These results indicate that CRAD knockdown-induced upregulation of the β -catenin reporter and target genes is due to β -catenin-mediated transcriptional activation.

To complement this, we also examined the effects of CRAD ectopic expression on Wnt/ β -catenin signaling in CRC cells. CRAD expression suppressed β -catenin reporter activity, *AXIN2*, and active β -catenin (Figs. 3h-3j). Moreover, the M1 and CPI constructs failed to downregulate *AXIN2* (Fig. 3k), suggesting that the CPI motifs in CRAD are required for suppression of β -catenin target gene activation.

Catenin proteins connect E-cadherin to the actin cytoskeleton, which contributes to the maintenance of epithelial cell integrity through cell-cell adhesion^{4,5,18,19}, and downregulates nuclear translocation of catenins²⁰. Given the role of CRAD in modulating the actin cytoskeleton (Fig. 2), we asked whether CRAD has an impact on the interaction between catenins and E-cadherin. In IECs, CRAD knockdown increased the levels of β -catenin and α -catenin both in the cytosol and the nucleus (Figs. 3l, S4c). Conversely, CRAD expression decreased nuclear β -catenin in HCT116 CRC cells (Figs. 3m, S4d). Moreover, co-IP assays showed that CRAD knockdown reduced the interaction between E-cadherin and catenin proteins (Figs. 3n, S4e). Conversely, in CRC cells, CRAD overexpression induced the binding of catenin proteins with E-cadherin (Figs. 3o, S4f). The Super Resolution microscopic analyses also showed that CRAD-expressing HCT116 cells displayed the increased β -catenin associated with E-cadherin at cell-cell adhesion (Figs. 3p, S4g). Since the actin cytoskeletal dynamics modulates E-cadherin-mediated cell adhesion^{5,18,21}, we asked whether CRAD-modulation of the actin cytoskeleton affects E-cadherin-catenin binding. Duolink (Fig. 3q) and co-IP assays showed that the ectopic expression of the FL and M2-M4 mutants stabilized the E-cadherin-catenin complex, while M1 and CPI mutants did not (Figs. 3r, 3s). We also checked β -catenin transcriptional activity under

conditions of actin-cytoskeletal inhibition *versus* stabilization. Actin polymerization inhibitors (Lat B, Cyto D) increased the transcriptional activity of β -catenin, while an F-actin stabilizer (Jasplakinolide) reduced it (Figs. S4h, S4i). These results suggest that under normal conditions, CRAD enhances the actin polymerization *via* CP inhibition, which stabilizes the CCA complex. Conversely, upon CRAD inactivation, a diminished actin cytoskeleton destabilizes the CCA complex. This releases β -catenin from the cadherin, followed by nuclear translocation of β -catenin and activation of Wnt/ β -catenin target genes (Fig. S4j).

Inhibition of CRC cell proliferation by CRAD

Having observed the inactivation of *CRAD* in CRC (see Fig. 1), and CRAD inactivation-induced Wnt/ β -catenin signaling activation, we next determined the effects of CRAD on IEC and CRC cell proliferation. Given high expression of CRAD in IECs, we depleted endogenous *CRAD* in IECs using shRNA. CRAD knockdown increased IEC proliferation (Fig. 4a), reversed by iCRT14 (Figs. 4b, S5a, S5b) or Eng-LEF1 (Figs. 4c, S5c-S5e). These data indicate that CRAD depletion-induced IECs hyperproliferation is mediated by β -catenin target gene activation. Conversely, CRAD overexpression inhibited CRC cell proliferation (Figs. 4d, S5f-S5h), which was rescued by β -catenin (Figs. 4e, S5i-S5o). These results suggest that CRAD-induced CRC cell growth inhibition is mainly due to the suppression of β -catenin signaling. Additionally, unlike the FL and M2-M4 mutants, the CPI and M1 constructs did not inhibit CRC cell proliferation (Figs. 4f, 4g, S5p, S5q). Importantly, CRAD depletion-induced IEC hyperproliferation was reverted by expression of FL, but not by expression of the CPI mutant (Fig. 4h). Similarly, β -catenin reporter activity was downregulated by the FL and M2-M4 whereas the CPI or M1 did not (Figs. 4i, 4j). These results suggest that the CPI motifs of CRAD are required to inhibit CRC cell proliferation.

Furthermore, xenograft transplantation assays showed that compared to HCT116, HCT116-CRAD injected mice exhibited the reduced tumor development (Figs. 4k, 4l), with notably decreased cell proliferation, increased F-actin, and downregulated β -catenin target (Figs. 4m-4o). These *in vitro* and *ex vivo* results suggest that CRAD inhibits CRC proliferation *via* suppression of β -catenin.

Intestinal adenoma development by *CRAD* KO

To address *in vivo* consequence of *CRAD* gene inactivation in CRC, we established a *CRAD* knockout (KO) mouse model (Figs. 5a, S6a-6d). Importantly, *CRAD* KO mice displayed adenoma development in the small intestine (Figs. 5b, 5c), in an age-dependent manner (Fig. 5d). Additionally, we found that *CRAD* KO mouse showed the decreased Wnt/ β -catenin target genes without the alteration of other signalings (Fig. S6e). Intriguingly, *CRAD* KO mice also developed pulmonary lesions resembling the early small cell lung cancer and solid pseudopapillary neoplasm (SPN) of the pancreas (Figs. S6f, S6g). These results are somewhat supported by the previous studies showing high mutation rates of *CRAD* in SCLC¹¹, and the constitutively active mutation of β -catenin in SPN of the pancreas²². Interestingly, mucin deposition in *CRAD* KO-induced intestinal adenoma was observed by

H&E staining and Periodic Acid-Schiff (PAS) staining (Figs. 5e, 5f). Moreover, *CRAD* KO tumors displayed the heterogeneous loss of epithelial cell integrity (H&E, CK19; Figs. 5e, 5g, S6h). The loss of epithelial cell integrity is known to induce mucosal secretion and intestinal inflammation²³. Of note, *CRAD* KO mice displayed the slightly increased intestinal inflammation (Figs. S6i, S6j). We also observed cell hyperproliferation in *CRAD* KO mice, indicated by the elevated number of Ki67- and phospho-Histone H3 (pHH3)-positive cells (Figs. 5h, S6k-S6n) without notable differences in cell death (Fig. S6o). Additionally, IHC results for lysozyme (Paneth cells) and chromogranin A (neuroendocrine cells) indicated an increase in Paneth cells and a decrease in neuroendocrine cells in the *CRAD* KO intestine (Figs. 5i, S6p), which might be because canonical Wnt signaling induces Paneth cell differentiation²⁴. Given that *CRAD* depletion activates Wnt/ β -catenin signaling by disrupting the CCA complex (see Fig. 3), we also examined cell-cell adhesion. *CRAD* KO mice showed the disorganized localization of E-cadherin and Villin (Figs. 5j, S6q). Additionally, β -catenin and its target genes were upregulated in *CRAD* KO mice (Figs. 5k, 5l, S6r). Also, *CRAD* KO-induced intestinal tumors exhibited the disorganized and decreased levels of F-actin (Fig. 5m). Moreover, compared to WT, intestinal extracts from the *CRAD* KO showed the delayed rate in actin polymerization (Fig. 5n). These results strongly suggest that *CRAD* KO *per se* is sufficient to initiate intestinal tumorigenesis with the loss of epithelial cell integrity and the aberrant activation of Wnt/ β -catenin signaling.

Accelerated intestinal tumorigenesis by *CRAD* heterogeneous KO

Given that *CRAD* gene mutations are often heterozygous in CRC patients (Fig. S7a), we asked whether genetic ablation of one allele of *CRAD* gene is associated with intestinal tumorigenesis. Compared to the *APC*^{MIN} strain, *APC*^{MIN}:*CRAD*^{+/-} mice exhibited an evident increase in tumor numbers in the small intestine (Figs. 6a-6c) without the change in β -catenin or cell proliferation (Figs. 6d-6f). Furthermore, unlike adenomas from *APC*^{MIN}, tumors of *APC*^{MIN}:*CRAD*^{+/-} displayed the loss of both F-actin (Fig. 6g) and epithelial cell properties (Fig. 6h), as observed in *CRAD* KO mice (see Figs. 5g, 5m).

Whereas *APC*^{MIN} mice barely develop tumors in the colorectum²⁵, *APC*^{MIN}:*CRAD*^{+/-} compound mice displayed severely invasive and mucinous adenomas in the colon (Figs. 6i-6k). Intriguingly, colonic tumors of *APC*^{MIN}:*CRAD*^{+/-} mice showed a markedly increased accumulation of mucin (Fig. 6l), similar to human mucinous colorectal carcinoma (MC)²⁶. Colonic adenomas from *APC*^{MIN}:*CRAD*^{+/-} mice also exhibited significant upregulation of β -catenin (Fig. 6m), Cyclin D1 (Figs. 6n, S7b, S7c), and cell hyperproliferation (Figs. 6o, S7d, S7e). Consistent with tumors in the small intestine, the heterogeneous loss of both F-actin and CK19 was also observed in colonic tumors of *APC*^{MIN}:*CRAD*^{+/-} mice (Figs. 6p, 6q). Moreover, we observed the invasive adenoma development in *APC*^{MIN}:*CRAD*^{+/-} mice, represented by the disruption of the basal membrane (Fig. S7f). Of note, both *APC*^{MIN} and *APC*^{MIN}:*CRAD*^{+/-} mice did not display the expression of mesenchymal markers (Fig. S7g), implying that epithelial-mesenchymal transition might not be involved in invasive tumor development in *APC*^{MIN}:*CRAD*^{+/-} mice. These results suggest that, in conjunction with *APC* inactivation, the deletion of *CRAD* allele leads to intestinal tumorigenesis in both the small and large intestine, which reveals a pathologic outcome of *CRAD* heterozygous mutation during intestinal tumorigenesis.

Mucinous Intestinal tumorigenesis by *CRAD* KO

We next examined the mucinous tumor phenotype driven by *CRAD* KO. Transformation or early tumor lesions can be assessed by development of the cystic spheroid organoid formation instead of the normal crypt organoids²⁷. Interestingly, *CRAD* KO developed the cystic spheroid as shown in *APC*^{MIN} organoids (Fig. 7a). *CRAD* KO cystic organoids exhibited the increased cell proliferation (Figs. 7b, S8a), stabilized β -catenin (Figs. 7c, S8b), upregulated β -catenin target genes (Figs. 7d-7f), disrupted actin cytoskeleton (Figs. 7g, 7h), abnormality of epithelial cell integrity (Fig. 7i), disorganized cell adhesion (Fig. 7j), and decreased IEC differentiation (Figs. 7k, 7l). *CRAD* KO cystic organoids also displayed the high expression of mucins (Figs. 8a, 8b) as shown in *CRAD* KO tumors (see Figs. 5e, 5f), indicating that *CRAD* KO upregulates mucin expression in a cell-autonomous manner. The goblet cells secrete various mucins, and the number of the goblet cells is increased in the MC^{28, 29}. Intriguingly, the non-tumor and tumor region of *CRAD* KO exhibited the increase in mucin expression (Figs. 8c, S8c) and the goblet cell number (Fig. S8d). qRT-PCR confirmed the marked upregulation of *mMUC* in *CRAD* KO tumors but not in *APC*^{MIN} tumors (Figs. 8c, S8e). Despite the implication of Wnt-Notch signaling axis in the goblet cell differentiation³⁰, only Wnt signaling target genes were upregulated by *CRAD* KO (Fig. S6e). MC is characterized by amplification of *TOP-1* (*Topoisomerase-1*) allele³¹. We found that the *CRAD* KO tumors exhibited the increase in both mRNA and genomic DNA levels of *TOP-1*, whereas *APC*^{MIN} tumors did not (Figs. 8e, 8f). Consistently, *CRAD* KO tumors and cystic spheroids also showed the upregulation of TOP-1 (Figs. 8g, S8f). Of note, TOP-1 upregulation (2mo; Fig. S8g) precedes mucin deposition (4mo; Figs. 8c, 8d, S8c). Furthermore, IHC of MC TMA showed the downregulation of CRAD expression in MC patient samples (Figs. 8h-8j). Oncomine datasets also indicated that *CRAD* expression is mutually exclusive to the expression of *MUC5B* and *MUC5AC* in MC (Fig. S8h). These data suggest that the loss or downregulation of CRAD is associated with the development of MC. Together, these results strongly suggest that the deletion of *CRAD* leads to the mucinous intestinal tumorigenesis.

Discussion

APC mutation in CRC causes aberrant Wnt/ β -catenin signaling activation. However, the β -catenin protein exhibits heterogeneous nuclear localization in the presence of the homogeneous mutation in *APC*^{32, 33}. This ' β -catenin paradox' model³²⁻³⁵ suggests that further activation of *APC* mutation-driven Wnt signaling contributes to intestinal tumorigenesis. In epithelial cells, APC competes with E-cadherin for β -catenin binding³⁶, suggesting that β -catenin might also be partially sequestered by E-cadherin in the *APC* mutated condition. Thus, it is likely that E-cadherin-mediated redistribution of β -catenin might be an additional layer limiting Wnt signaling in normal tissues.

Our study proposes that the interaction between the E-cadherin-catenin complex and the actin cytoskeleton might be a key factor suppressing tumorigenesis. Similarly, during embryogenesis, cadherin antagonizes β -catenin activity³⁷. Although E-cadherin and APC might also be potential candidate regulators for CCA complex in tumorigenesis, E-cadherin mutation rate is meager in CRC³⁸ and APC's function is inhibited by CPs³⁹. Therefore, it is

plausible that the defects in other key regulators of the CCA complex might be implicated in CRC. Our findings that *CRAD* ablation-induced F-actin depolymerization leads to intestinal tumorigenesis strongly suggest that *CRAD*-modulated actin cytoskeletal dynamics and CCA complex stabilization plays a crucial tumor suppressive role in the intestinal epithelium.

The limitation of *APC^{MIN}* mouse model is that the development of intestinal adenomas mainly occurs in the small intestine not frequently in the colorectum, unlike human CRC²⁵. *CRAD* KO mice develop tumors in both the small and large intestine (Fig. 6), which somewhat recapitulates human CRC pathology. Moreover, the *APC^{MIN};CRAD^{+/-}* mice display microinvasion without EMT (Figs. S7f, S7g), implying the potential roles of *CRAD* inactivation in CRC metastasis.

MC (10–15% of human CRC) is highly metastatic and therapeutically resistant³¹. However, the molecular mechanism of MC development remains elusive. Tumors developed from *CRAD* KO mice display the excessive mucin deposition (Figs. 5e, 5f, 6j, 6l) with *TOP-1* upregulation (Figs. 8e, 8f), mimicking human MC. This is further supported by the downregulation of *CRAD* in human MC (Figs. 8h–8j). Of note, *APC^{MIN}* mice do not develop mucinous adenoma, implying that *CRAD* loss-induced MC development process might include distinct pathologic events in addition to Wnt/ β -catenin hyperactivation. In normal intestine, mucin secreted by goblet cells is primarily involved in innate host defense. However, an abnormally elevated secretion of mucin contributes to CRC progression⁴⁰. Thus, *CRAD* inactivation might also contribute to tumorigenesis *via* high mucin secretion, beyond Wnt signaling. It is noteworthy that *CRAD* KO cystic organoids display mucin upregulation in a cell-autonomous manner (Figs. 8a, 8b), excluding the potential involvement of immunocytes in mucin expression. Importantly, given no precedent MC mouse model, our *CRAD* KO mice may be highly beneficial to studies of human MC etiology.

Together, our study reveals that *CRAD* is a tumor suppressor and indispensable for the maintenance of epithelial cell integrity through modulation of the cytoskeleton and thereby CCA complex.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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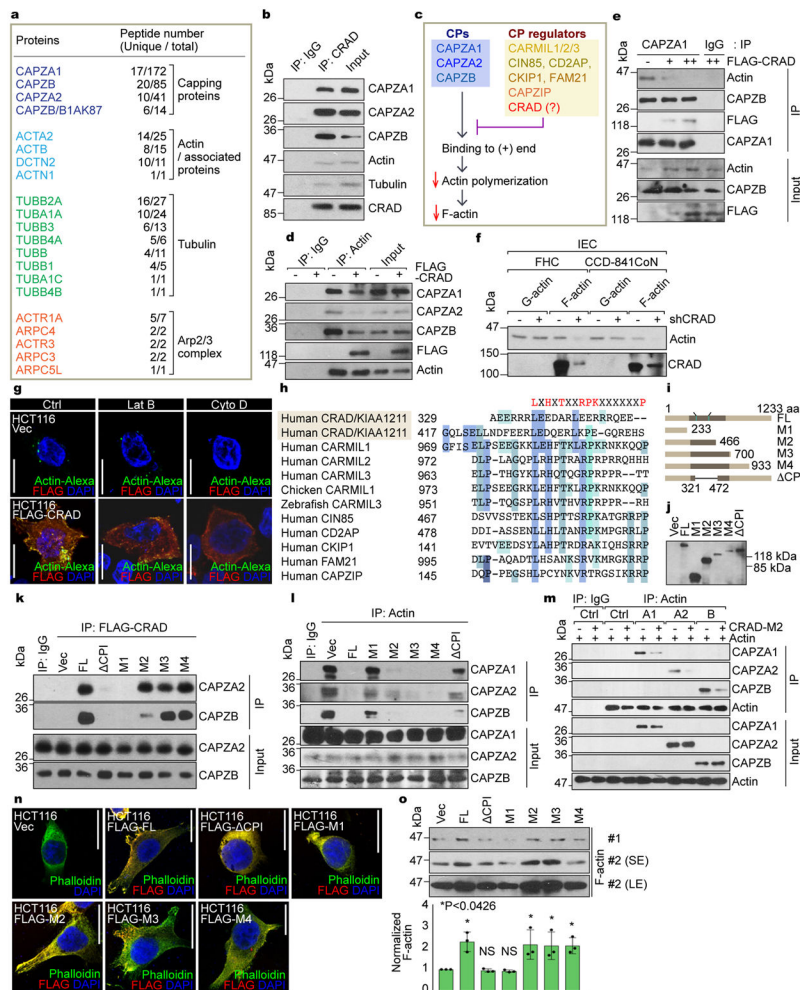


Figure 2. Positive regulation of the actin polymerization by CRAD-inhibited capping proteins
a, CRAD-interacting proteins identified by tandem affinity purification and mass spectrometry (TAP-MS) (see Table S2). TAP-MS was performed once.
b, The endogenous interaction of CRAD with CPs, actin, and tubulin. FHC cell lysates were analyzed for co-IP.
c, Illustration of the hypothetical model of CRAD-induced actin polymerization.
d and e, The decreased interaction between CPs and actin by CRAD. The reciprocal co-IP analysis of HCT116 cells transfected with FLAG-CRAD plasmid, with either actin (**d**) or CAPZA1 antibodies (**e**).
f, Decreased F-actin by CRAD depletion in IECs. Fractionation and IB assays of F-/G-actin.
g, The increase of uncapped barbed (+) ends by CRAD. Cells were permeabilized by the saponin-containing buffer for visualization of uncapped barbed (+) ends using Super Resolution microscope. Images are representative of two independent experiments (n=3 each independent samples) with similar results.
h, Comparative amino acid sequence analysis of potential CPI motifs in CRAD with those in known CP regulators.
i and j, The generation of CRAD mutant constructs (**i**) and IB assays (**j**). IB was performed once.

k and l, CRAD-CPs binding *via* CPI motifs. The reciprocal co-IP analysis of HCT116 cell lysates transfected with FLAG-CRAD (FL, CPI, and M1-M4) plasmids, with either FLAG (**k**) or actin antibodies (**l**).

m, The decreased interaction between CAPZs and actin by CRAD. Direct binding and blocking were analyzed by co-IP assay using purified recombinant proteins.

n and o, The increase of F-actin formation by ectopic expression of CPI motifs-containing CRAD mutants. After 24hr transfection with each plasmid, HCT116 CRC cells were visualized by Phalloidin IF staining (**n**). Images are representative of three independent experiments (n=3 each independent samples) with similar results. Cells were also fractionated into F-actin and G-actin and analyzed for IB (**o**; upper), (normalized by G-actin expression using ImageJ [**u**; lower]). SE/LE: short or long exposure.

Scale bars indicate 20 μ m; Data in panels b, d-f, k-m, and o are from n=3 independent experiments; Error bars: mean \pm S.D.; NS: not significant ($P>0.05$); Two-sided unpaired *t*-test.

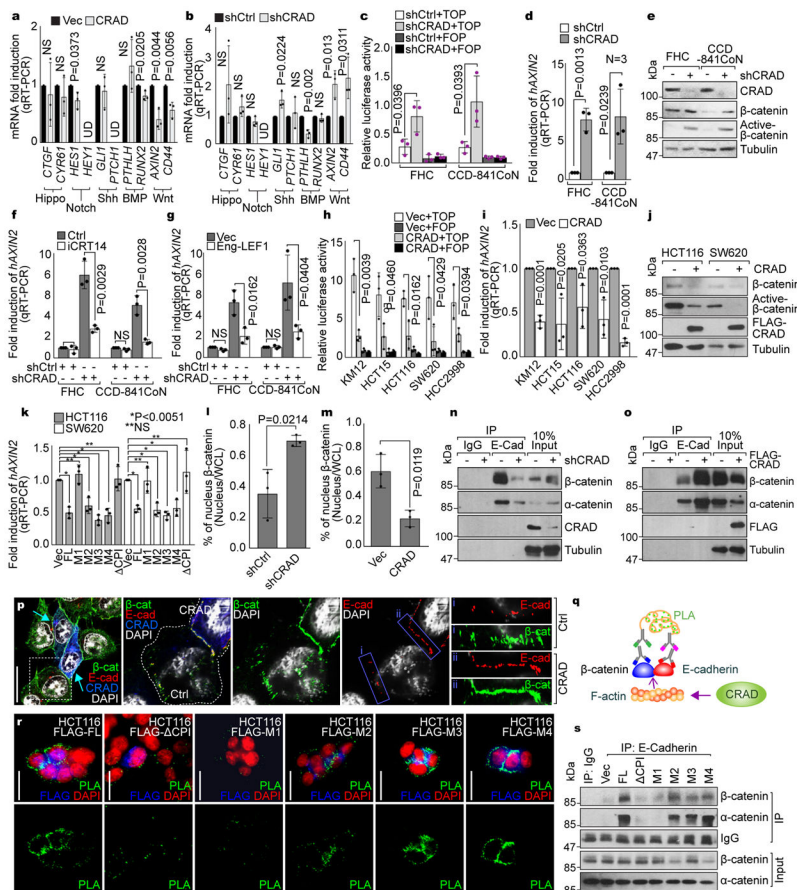


Figure 3. Loss of CRAD-activated Wnt signaling by disrupting CCA complex

a, Decreased Wnt signaling target genes by CRAD. 24hr after transfection, HCT116 cells were analyzed for qRT-PCR.

b, Increased Wnt signaling target genes by CRAD knockdown. CRAD-depleted CCD-841CoN cells were analyzed for qRT-PCR.

c and d, Increased β-catenin transcriptional activity by CRAD depletion. IECs were transfected with β-catenin reporter plasmids (TOP/FOPFLASH) for luciferase assays (**c**). qRT-PCR for *AXIN2* (**d**).

e, Increased β-catenin protein by CRAD depletion in IECs. IB assays.

f and g, Inhibition of CRAD depletion-induced *AXIN2* upregulation by iCRT14 (**f**) or Eng-LEF1 (**g**). 24hr after iCRT14 (an inhibitor of β-catenin-TCF binding; 100μM) treatment or Eng-LEF1 (a dominant-negative mutant blocking β-catenin-mediated gene activation) transient transfection, IECs were analyzed for qRT-PCR.

h-j, Suppression of β-catenin transcriptional activity by CRAD in CRC cells. 24hr after transfection, CRC cells were analyzed for TOP/FOPFLASH luciferase analysis (**h**), qRT-PCR of *AXIN2* (**i**), and IB for β-catenin (**j**). Experiment performed once.

k, The inhibition of β-catenin target gene expression by CPI motif-containing CRAD mutants. 24hr after transfection, CRC cells were analyzed for TOP/FOPFLASH luciferase activity.

l, Decreased nuclear β -catenin by CRAD. IECs (**l**) and CRC cells (**m**) were transfected with shCtrl or shCRAD and Vec or CRAD, respectively. After 48hr, cells were fractionated into the cytosolic and nucleus fractions, followed by IB. Quantification of nucleus β -catenin was assessed using ImageJ.

n, Decreased interaction between E-cadherin and catenins by CRAD depletion. Co-IP assays of shCRAD-CCD-841CoN. The representative images are shown from three independent experiments with similar results.

o and p, Increased interaction between E-cadherin and catenins by CRAD. HCT116 cells were transfected with FLAG-CRAD plasmid. Co-IP assays (**o**) and IF staining (**p**). Arrows indicate CRAD-expressing cells. Compared to i (non-transfected cells), ii (CRAD-expressing cells) displays the increased colocalization of E-cadherin and β -catenin by CRAD. The representative images are shown from three independent experiments with similar results.

q, Illustration of E-cadherin- β -catenin binding analysis using Duolink assays.

r and s, Restoration of E-cadherin- β -catenin binding by CPI motif-containing CRAD mutants in CRC cells. Duolink assay (**r**). Green (PLA) fluorescence indicates E-cadherin- β -catenin interaction. Co-IP analysis (**s**).

Representative images of three experiments with similar results; Scale bars indicate 20 μ m; Data in panels a-h and k-m are from n=3 independent experiments; Error bars: mean \pm S.D.; NS: not significant ($P>0.05$); Two-sided unpaired *t*-test;

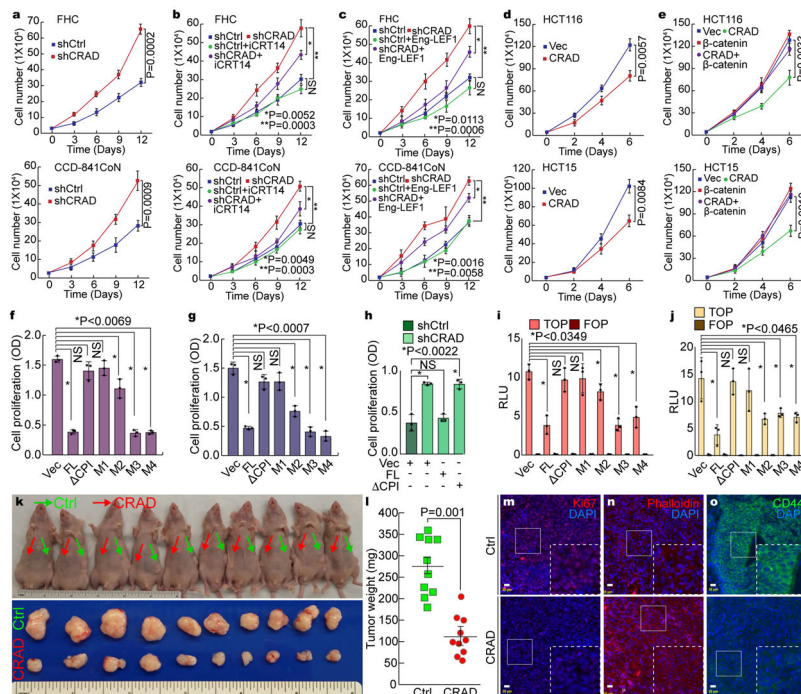


Figure 4. Inhibition of CRC cell proliferation by CRAD

a, IEC hyperproliferation by CRAD depletion. The proliferation of FHC and CCD-841CoN cells (shCtrl [control] and shCRAD) were analyzed by cell counting.

b and c, Suppression of shCRAD-induced cell hyperproliferation by β -catenin inhibition in IECs. FHC and CCD-841CoN (shCtrl and shCRAD) cells were treated with iCRT14 (100 μ M) for 14 days, and cell number was counted (**b**). IECs (shCtrl and shCRAD) were transfected with Eng-LEF1 and analyzed for cell proliferation (**c**).

d, CRC cell growth inhibition by CRAD expression. HCT116 and HCT15 cells (Vec [control] and CRAD expressing) were analyzed for cell proliferation.

e, β -catenin rescues CRAD-induced CRC cell growth inhibition. HCT116 and HCT15 cells were transfected with CRAD or β -catenin plasmids and analyzed for cell proliferation.

f-h, CRC cell growth inhibition by CPI motif-containing CRAD mutants. CRAD (FL, CPI, and M1-M4)-transfected CRC cells were analyzed for cell proliferation. HCT116 (**f**); SW620 cells (**g**). CCD-841CoN cells were transfected with each plasmid and analyzed for cell proliferation (**h**).

i and j, Suppression of β -catenin reporter by CPI motif-containing CRAD mutants. HCT116 (**i**) and SW620 (**j**) cells transfected with CRAD FL or mutant constructs were analyzed for luciferase activity.

k-o, Inhibition of *ex vivo* tumor development by CRAD. HCT116 (control [Ctrl]) and HCT116-CRAD cells were subcutaneously injected into the left flank (control; green arrows; **k**) and the right flank (CRAD-expressing; red arrows; **k**), and analyzed for tumor weight (**l**; n=10 mice) and IHC (**m-o**); Ki67 (**m**); Phalloidin (**n**); CD44 (**o**). These experiments (**k and l**) were performed once.

Scale bars indicate 20 μ m; Data in panel a-j and o are from n=3 independent experiments; Error bars: mean \pm S.D.; NS: not significant ($P>0.05$); Two-sided unpaired *t*-test.

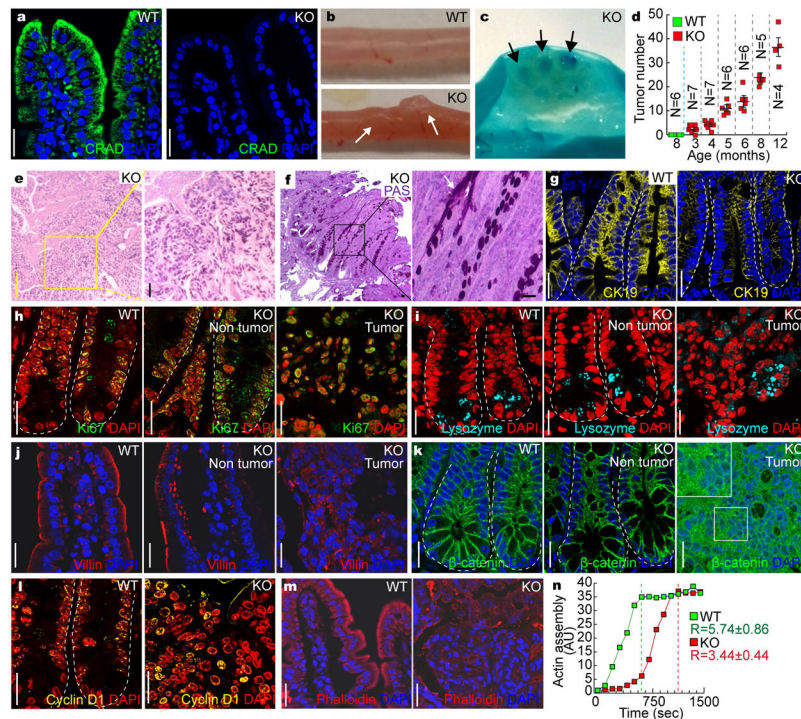


Figure 5. Intestinal adenoma development by *CRAD* KO

a, *CRAD* expression in the small intestine. Immunohistochemistry (IHC) of mouse intestine. *CRAD* KO mouse serves as a negative control.

b and c, Intestinal adenoma development in *CRAD* KO mice. The adenomas in the small intestine of *CRAD* KO mice (3mo of age; **b**). Methylene blue staining (**c**). Arrows indicate intestinal adenoma.

d, Age-dependent intestinal adenoma development in *CRAD* KO mice. N values indicate the number of mice. Error bars: mean \pm S.D. The experiment was performed once.

e, Hematoxylin and eosin (H&E) staining of intestinal adenoma (*CRAD* KO).

f, Periodic Acid-Schiff (PAS) staining of intestinal adenoma in *CRAD* KO mice.

g, Disruption of epithelial cell integrity. Cytokeratin 19 (CK19). Arrows: Villi not expressing CK19.

h, Cell hyperproliferation in *CRAD* KO small intestine. *CRAD* KO Ki67.

i, Abnormal differentiation of IECs by *CRAD* KO. WT and *CRAD* KO small intestine were immunostained with Lysozyme.

j, Disorganized cell adhesion in *CRAD* KO mice. Cells were stained with Villin.

k, The increase of β -catenin in *CRAD* KO tumor.

l, Upregulation of β -catenin target genes in the intestinal adenoma of *CRAD* KO mice. IHC for Cyclin D1.

m, Disorganized actin cytoskeleton in *CRAD* KO-induced tumor. F-actin was visualized by Phalloidin staining.

n, The decrease of the actin polymerization in *CRAD* KO mice. Cell extracts from the small intestine were analyzed for actin polymerization assays. n=3 independent experiments. R values indicate the velocity of actin assembly.

Representative images of three independent mice per group (WT vs. KO); AU: arbitrary unit; Scale bars indicate 20 μ m.

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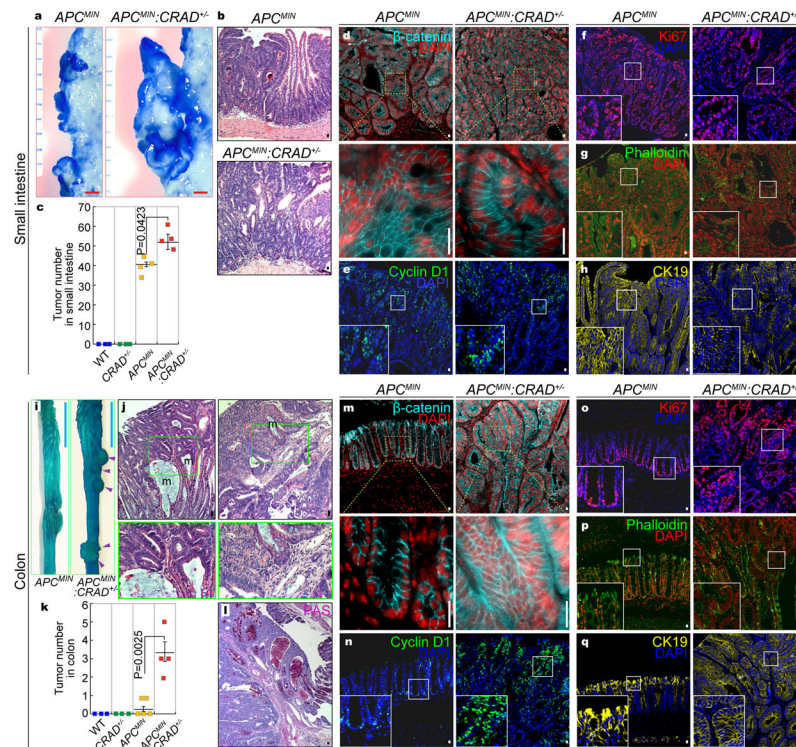


Figure 6. Accelerated intestinal tumorigenesis by *CRAD* heterogeneous KO

a, Representative images of intestinal tumors from the small intestine of APC^{MIN} (n=4 mice) and $APC^{MIN};CRAD^{+/-}$ (n=4 mice) (4mo of age).

b and c, The increase of small intestinal tumors in $APC^{MIN};CRAD^{+/-}$ (n=4) mice, compared to APC^{MIN} (n=4) mice. Representative H&E images of small intestinal tumors in APC^{MIN} and $APC^{MIN};CRAD^{+/-}$ mice (**b**). Quantification of adenomas (4mo of age; **c**).

d-h, IHC of intestinal tumors from the small intestine of APC^{MIN} and $APC^{MIN};CRAD^{+/-}$ mice. β -catenin (**d**); Cyclin D1 (**e**); Ki67 (**f**); Phalloidin (**g**); CK19 (**h**).

i, Colorectal tumors in $APC^{MIN};CRAD^{+/-}$ (n=4) mice (4mo of age; Arrowheads).

j, H&E staining of the colorectal tumors in $APC^{MIN};CRAD^{+/-}$ mice. m: mucin-accumulated lesion. 4mo of age. n=3.

k, Comparative analysis of colorectal tumors (4mo of age). WT (n=3 mice); $CRAD^{+/-}$ (n=3 mice); APC^{MIN} (n=5 mice); $APC^{MIN};CRAD^{+/-}$ (n=4 mice.)

l, PAS staining of colorectal tumors in $APC^{MIN};CRAD^{+/-}$ mice.

m-q, IHC of colorectal tumors from APC^{MIN} and $APC^{MIN};CRAD^{+/-}$ mice (4mo of age). β -catenin (**m**); Cyclin D1 (**n**); Ki67 (**o**); Phalloidin (**p**); CK19 (**q**).

Images of panel b, d-h, j, and l-q are representative of IHC experiments from three independent tumors; Red scale bars indicate 1mm; Blue scale bars indicate 10mm; Black or white scale bars indicate 20 μ m; Error bars: mean \pm S.D.; Two-sided unpaired *t*-test.

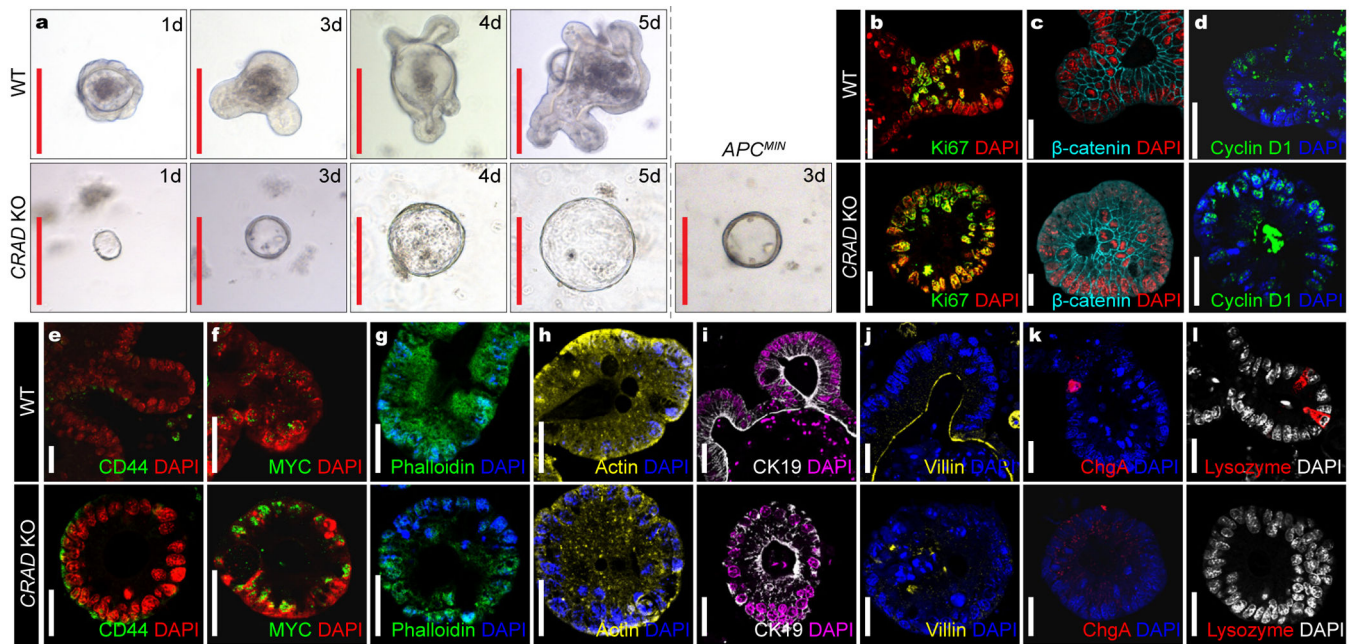


Figure 7. Mucinous Intestinal tumorigenesis by *CRAD* KO

a, Cystic spheroids formation by *CRAD* KO. Isolated crypts from WT, *CRAD* KO, and *APC*^{MIN} were maintained in the organoid culture medium. These data are representative of three independent organoid experiments with similar results. 10 organoids per group [WT vs. KO] were analyzed.

b-l, IHC analysis of the organoids derived from *CRAD* WT and KO mouse intestine. Compared to WT, *CRAD* KO-driven cystic spheroids showed that increase of cell proliferation (Ki67; **b**), increase of β-catenin (**c**) and its target genes (Cyclin D1 [**d**]; CD44 [**e**]; MYC [**f**]), disruption of the actin cytoskeleton (Phalloidin [**g**]; Actin [**h**]), loss of epithelial cell integrity (CK19 [**i**]; Villin [**j**]), and decreased IEC lineage differentiation (Chromogranin A: ChgA; [**k**]; Lysozyme [**l**]).

Representative images of three experiments; Red scale bars indicate 20 μm; White scale bars indicate 20 μm.

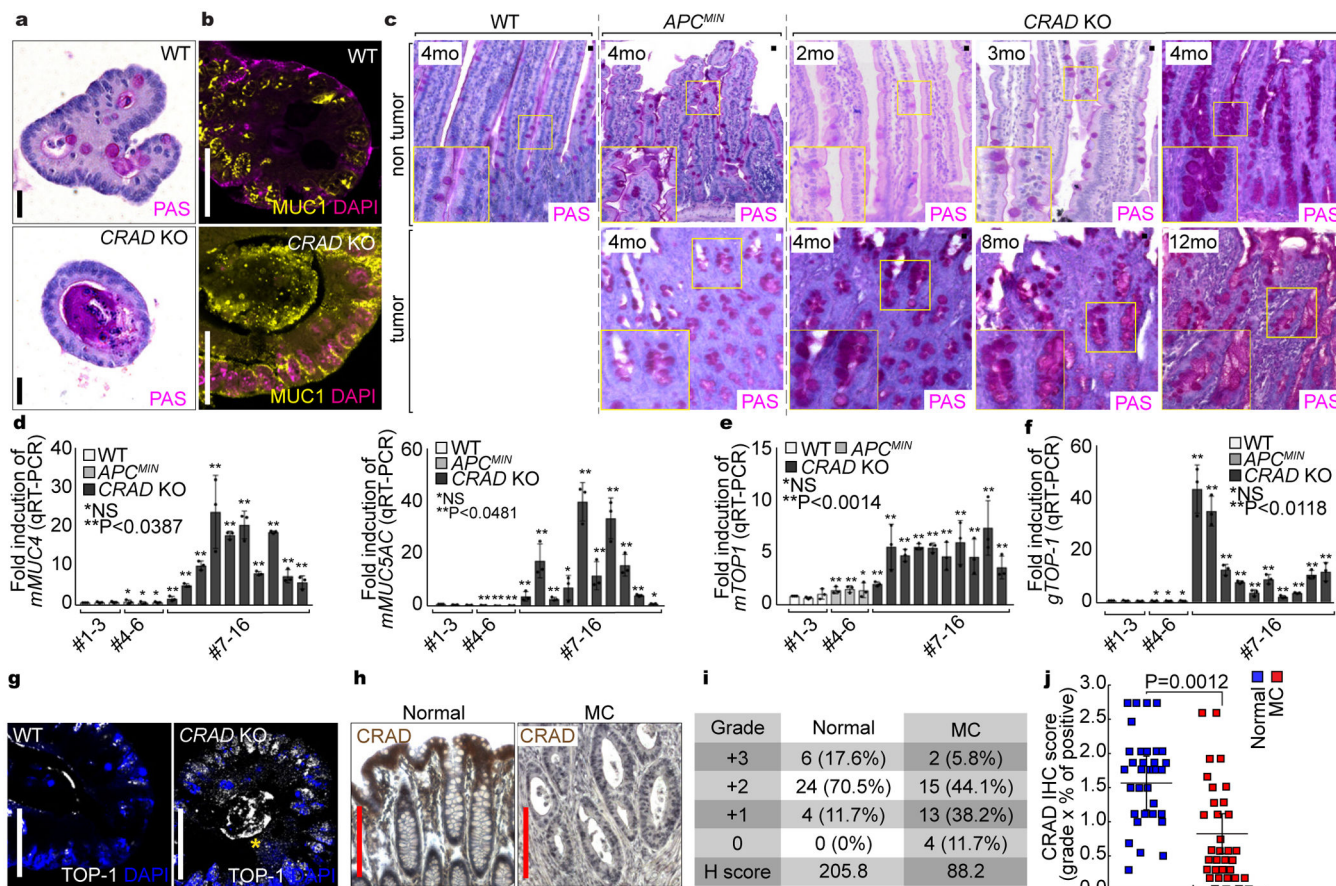


Figure 8. Increased mucin deposition by CRAD KO

a and b, Excessive mucin deposition in CRAD KO-induced cystic spheroids. PAS staining (a) and IHC of MUC1 (b) were performed using organoids from WT and CRAD KO mice.

c and d, Increased mucin deposition in CRAD KO-induced tumors. After fixation and paraffin embedding, each sample was stained with PAS (c). Increased mucin expression in CRAD KO tumors (qRT-PCR; d).

e-g, Upregulation of TOP-1 in CRAD KO tumors. WT intestine (#1-3) and tumors from APC^{MIN} (#4-6) and CRAD KO (#7-16) were analyzed for TOP-1 mRNA (qRT-PCR; e; n=3) and genomic DNA (real-time PCR; f). After 5 days of culture, normal crypt organoid from CRAD WT and spheroid organoids from CRAD KO were immunostained with a TOP-1 antibody (g).

h-j, CRAD inactivation in MC patients. IHC of TMA with CRAD antibody, Images are representative of 34 patients samples (h). After scoring of CRAD expression, H-scores (i) and IHC scores (j) were calculated. Normal (n=34 patient samples) vs. MC (n=34 patient samples).

Images of panel a-c and g are representative of three independent experiments; Red scale bars indicate 200µm; black or white scale bars indicate 20µm; Data in panel d-f were obtained from n=3 independent experiments; Error bars: mean ± S.D.; NS: not significant (P>0.05); Two-sided unpaired t-test.