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E2F1 represses β -catenin transcription and is antagonized by both pRB and CDK8

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Abstract

The E2F1 transcription factor can promote proliferation or apoptosis when activated, and is a key downstream target of the retinoblastoma tumor suppressor protein (pRB). Here we show that E2F1 is a potent and specific inhibitor of β -catenin/T-cell factor (TCF)-dependent transcription, and that this function contributes to E2F1-induced apoptosis. E2F1 deregulation suppresses β -catenin activity in an adenomatous polyposis coli (APC)/glycogen synthase kinase-3 (GSK3)-independent manner, reducing the expression of key β -catenin targets including *c-MYC*. This interaction explains why colorectal tumors, which depend on β -catenin transcription for their abnormal proliferation, keep *RB1* intact. Remarkably, E2F1 activity is also repressed by cyclin-dependent kinase-8 (CDK8), a colorectal oncoprotein. Elevated levels of CDK8 protect β -catenin/TCF-dependent transcription from inhibition by E2F1. Thus, by retaining *RB1* and amplifying *CDK8*, colorectal tumor cells select conditions that collectively suppress E2F1 and enhance the activity of β -catenin.

Keywords

apoptosis; E2F1; β -catenin; CDK8; pRB; retinoblastoma; TCF; Wnt

E2F1 is generally dispensable for cell proliferation but is selectively activated in response to specific cues, such as DNA damage, where it drives the expression of pro-apoptotic genes. Ectopic expression of *Drosophila E2F1* (*dE2f1*) in the developing wing causes apoptosis, giving a visible, dosage-sensitive phenotype that we have used to screen for *in vivo* regulators of E2F-dependent apoptosis². Using this strategy, we found a novel interaction between *dE2f1* and the Wnt signaling pathway (Fig. 1). An apoptotic, gnarled wing phenotype (Fig. 1b), caused by elevated *dE2f1* in newly-eclosed wing epithelial cells, was strongly suppressed by co-expression of the *Drosophila* β -catenin ortholog *armadillo* (*arm*) (Fig. 1d), and partially suppressed by co-expression of *pangolin* (*pan*) (Fig. 1e), which encodes *dTCF*, the transcription factor partner of *arm*. Moreover, expression of dominant-

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negative, amino-terminally truncated *pan* (*dTCF ΔN*) phenocopied the *dE2f1* wing phenotype (Fig. 1f), and ectopic expression of *shaggy* (*sgg*; the GSK3 ortholog and negative regulator of Arm protein stability) strongly enhanced the effects of *dE2f1* (Fig. 1g, h). Using a stable and activated-mutant form of *arm* (*arm**; S44Y mutation in *sgg*/GSK3 phosphorylation site3), we tested whether *dE2f1* expression could modify an *arm*-dependent phenotype. Expression of *arm** under the *GMR* eye specific promoter causes a rough eye phenotype (Fig. 1k) that was partially suppressed by co-expression of *dE2f1/dDp* (Fig. 1l). Together, these genetic interactions shows a strong functional antagonism between elevated dE2F1/dDP and Arm/β-catenin signaling *in vivo*.

Wnt/β-catenin signaling is important during development and regulates diverse aspects of cell function including proliferation, differentiation and survival^{4,5}. The genetic interactions suggested that E2F1 might inhibit Arm/β-catenin-dependent transcription. To test this, and to ask whether the interaction was conserved in human cells, we examined the effects of E2F1 on activation of a TCF-luciferase reporter (pTopFLASH) by a stable, tumor-derived form of β-catenin (S33Y6). In human Saos2 cells, a *p53*- and *Rb*-deficient cell line that exhibits low basal Wnt activity, expression of E2F1 strongly inhibited S33Y-β-catenin transcription (Fig. 1m). Control experiments showed that the inhibition was not an indirect consequence of E2F1-induced apoptosis or cell cycle progression (Fig. 1 and Supplementary Fig. 2). Inhibition by E2F1 was comparable to the effects of dominant-negative forms of TCF1 or TCF4, enhanced by co-expression of the E2F1-dimerization partner DP1, and abrogated by mutation of DP1- or DNA-binding domains of E2F1 (Supplementary Fig. 2 and Supplementary Fig 3). Remarkably, as little as 10 ng of E2F1 expression plasmid repressed pTopFLASH activity ten-fold when co-transfected with DP1 (Supplementary Fig. 2c). E2F1 activated transcription from a canonical E2F-luciferase reporter (pE2F4B) in these cells (Fig. 1m, n), indicating that it has context-dependent effects. Inhibition of β-catenin was a specific property of E2F1, as other activator E2Fs had little effect (Fig. 1n). Using E2F1/3 chimeras⁷, we mapped the inhibitory region to the Marked Box and adjacent domains of E2F1 (Supplementary Fig. 3), regions that allow selective interactions with other transcription factors and that determine the differences between the transcriptional signatures of E2F1 and E2F38.

The pro-apoptotic activity of E2F1 has been linked to the *TP53* and *TP73* tumor suppressors; intriguingly, both of these also affect β-catenin-dependent transcription from the pTopFLASH reporter^{9,10,11}. *p53*, like E2F1, inhibited pTopFLASH transcription, whereas *p73*, a *p53*-related gene that is a transcriptional target of E2F1, activated pTopFLASH (Fig. 1o). In *TP53*-deficient Saos2 cells, E2F1 repressed β-catenin-mediated pTopFLASH activity and dominantly suppressed the stimulatory effects of *p73* (Fig. 1o). Thus, E2F1 is a potent inhibitor of β-catenin/TCF activated transcription that acts independently of *p53* and is dominant over *p73*.

To determine the effects of E2F1 on endogenous β-catenin-regulated genes we first examined *c-MYC*, one of the best-studied targets of β-catenin and a key mediator of the proliferative effects of deregulated β-catenin during tumorigenesis¹². Levels of *c-MYC* mRNA and protein rapidly decreased following E2F1 induction in Saos2-*TR-E2F1* cells (Fig. 2a, c). *PPARδ* and *CD44*, two other well-studied Wnt targets showed similar changes (Fig. 2c). As a control, E2F1 strongly activated the well known E2F targets genes, *CCNE1* (*Cyclin E*) and *TP73* (Fig. 2b). Similar effects on Wnt target genes were observed when E2F1 was expressed in colorectal cancer cells (Fig. 2f).

The list of known Wnt targets includes genes that control β-catenin degradation, such as *AXIN1* and *AXIN2*. Interestingly, the expression of *AXIN1* and *AXIN2*, as well as *SIAH1*, a *p53*-inducible, GSK3-independent promoter of β-catenin degradation^{13,14}, were all

significantly activated by E2F1 (Fig. 2d, e), consistent with previous studies suggesting that *AXIN2* and *SIAH1* are E2F-target genes^{15,16}. Accordingly, the level of β -catenin protein decreased at later time points following E2F1 expression, a change that preceded apoptosis (Fig. 2g and Supplementary Fig. 4). Similarly, ectopic expression of *Drosophila* dE2F1/dDP reduced Arm protein levels (Supplementary Fig. 4h).

The mechanism of E2F1-dependent β -catenin downregulation is likely to be distinct from the changes observed during epithelial-mesenchymal transition, or following the disruption of adherens junctions or focal-adhesions, as markers for these processes were unperturbed by E2F1 expression (Supplementary Fig. 4i). Instead, E2F1 induced the post-translational degradation of β -catenin in a GSK3- and caspase-independent fashion (Fig. 2h and Supplementary Fig. 4g). E2F1-mediated degradation of β -catenin is functionally significant since re-expression of stable, tumor-derived mutants of β -catenin, or treatment with GSK3-inhibitors, partially abrogated E2F1-dependent apoptosis (Fig. 2i). Taken together, these results show that E2F1 inhibits β -catenin activity via transcriptional antagonism and β -catenin degradation, and that this inhibition contributes to E2F1-induced apoptosis.

β -catenin-dependent transcription is crucially important for cell proliferation in colorectal cancer cells. Mutations in *APC* or *CTNNB1* (β -catenin) occur early in colorectal tumorigenesis, leading to pre-malignant polyps. Additional mutations contribute to the transition to malignant adenocarcinoma^{4,5}. An unusual feature of colorectal cancer cells is that they rarely (if ever) acquire mutations in the *RBI* tumor suppressor gene. Paradoxically, *RBI* copy gains are frequently found in colorectal cancer cells, often resulting in protein overexpression¹⁷. Conditional inactivation of murine *Rb* by *Villin-Cre* leads to aggressive tumors in various tissues, but rarely in the GI tract^{18,19}, and the knockdown of pRB reduces cell proliferation and anchorage-independent growth of human colon cancer cell lines²⁰. Accordingly, we found elevated levels of pRB and β -catenin co-localized within the epithelium of *Apc^{Min}* colonic tumors in mice (Fig. 3a, b). We hypothesized that colorectal tumor cells might select for mechanisms that limit the activity of E2F1, and that in this context the pRB tumor suppressor might act to sustain high levels of β -catenin/TCF-dependent transcription.

To test this hypothesis, we utilized a stable line of U2OS osteosarcoma cells containing a doxycycline (Dox)-inducible short-hairpin RNA targeting *Rb* (U2OS-*shRb*)²¹. Depletion of pRB increased transcription from an E2F reporter and inhibited transcription from the pTopFLASH reporter (Fig. 3c, d). In SW480 colorectal cancer cells that contain mutant *APC* and have deregulated β -catenin²², the expression of E2F1 sufficed to activate the E2F-luciferase reporter and to inactivate basal pTopFLASH transcription (Fig. 3e). Moreover, a shRNA vector that targets pRB inhibited the activity of endogenous β -catenin/TCF (Fig. 3f) and strongly inhibited cell proliferation (Fig. 3g), an effect partially rescued by co-expression of *S33Y- β -catenin/TCF*, but not *Bcl-2* (Fig. 3g). Together, these observations provide a molecular explanation for why colorectal tumor cells maintain the expression of pRB.

Because E2F1 is a potent inhibitor of β -catenin, we reasoned that tumor cells might select for additional ways to limit its activity. We generated transgenic flies that allowed us to knock-down dE2F1 in a tissue-specific manner (*dE2f1^{RNAi}*; Fig. 4 and Supplementary Fig 5). This approach reduced dE2F1 activity *in vivo* and generated phenotypes that were used to screen for factors that were rate-limiting for dE2F1-dependent proliferation *in vivo* (see Methods). We identified *dCdk8^{c01804}*, a hypomorphic mutant allele of *Drosophila Cdk8*, as a strong and specific suppressor of *dE2f1^{RNAi}* phenotypes in both the eye and wing (Fig. 4a-c and Supplementary Fig. 5). CDK8, Cyclin C, MED12 and MED13 form a sub-module of the Mediator complex, a large multi-subunit regulator of transcription^{23,24}. We observed

increased expression of dE2F1-regulated genes in *dCdk8* or *dCycC* mutant larvae (Fig. 4d). RNAi-mediated depletion of dCDK8 or dCycC in *Drosophila* SL2 cells caused similar changes (Supplementary Fig. 6a). In addition, RNAi-mediated depletion of dCDK8, or other components of the CDK8 sub-module, partially suppressed the cell proliferation defects caused by depletion of dE2F1 (Supplementary Fig. 6b–c).

GST-pulldown assays demonstrated a strong physical interaction between dCDK8 and dE2F1 (Fig. 4e) that mapped to the dE2F1 transactivation domain (Supplementary Fig. 7). The physical interaction between E2F1 and CDK8 is conserved between species: human E2F1 co-immunoprecipitated with CDK8 from Saos2 cell extracts (Fig. 4f). Moreover, E2F1 was specifically phosphorylated by CDK8 when complexes were incubated in kinase buffer (Fig. 4g). Thus, CDK8 physically interacts with E2F1 and is a conserved negative regulator of E2F1-dependent transcription.

The interaction between CDK8 and E2F1 is particularly notable, as a concurrent study has found significant *CDK8* copy number gains in colorectal cancers; approximately 40% of tumors have copy number gains in both *CDK8* and *RB1*. Chromatin immunoprecipitation (ChIP) experiments on SW480 colorectal cancer cells confirmed that CDK8 and E2F1 are both present at E2F-regulated promoters as well as the *c-MYC* promoter (Supplementary Fig. 8), suggesting an interplay between E2F1, CDK8, and β -catenin/TCF. In Saos2 cells, which contain low basal Wnt activity, expression of CDK8 enhanced β -catenin activation from the pTopFLASH reporter, while expression of kinase-dead CDK8 (CDK8KD) had no effect (Fig. 4h). Moreover, expression of CDK8, but not CDK8KD, suppressed the inhibitory effect of E2F1 on the pTopFLASH reporter (Fig. 4h). Similar results were observed for CDK8 expression in HCT116 colorectal cancer cells (Supplementary Figure 8). Hence, CDK8 and E2F1 have antagonistic effects on β -catenin-mediated transcription, and increasing the levels of CDK8 protects β -catenin from inhibition by E2F1. In agreement with this, the expression of CDK8KD reduced pTopFLASH activity in APC-deficient SW480 colorectal cancer cells, and enhanced the inhibition caused by short-hairpin targeting of pRB (Figure 4i).

Cancer cells acquire multiple mutations during tumorigenesis and it is a major challenge to explain how each change contributes to malignancy. However, the absence of mutations can also give new insights. It has long been known that colorectal tumor cells fail to mutate *RB1* and typically express elevated levels of this tumor suppressor. The discovery that E2F1 is a potent inhibitor of β -catenin-dependent transcription provides an unexpected and simple explanation to this conundrum. This interaction may also explain why colorectal tumors frequently overexpress c-myc-induced micro-RNAs that target E2F125–26. The discovery by Firestein and colleagues¹ showing significant *RB1* and *CDK8* copy number gains in colorectal cancers is especially intriguing given the evidence that CDK8 is an important modulator of both β -catenin and E2F1. Whereas CDK8 enhances the activity of β -catenin, it represses the activity of E2F1. Consequently, the amplification of *CDK8* may act as a switch, allowing increased β -catenin-dependent transcription that is also resistant to E2F1 inhibition (see model in Supplementary Fig. 1). Reversing this process, such as the inhibition of CDK8 combined with the activation of E2F1, may be useful as a two-pronged strategy to target cancer cells that are driven by deregulated β -catenin activity.

METHODS SUMMARY

Unless otherwise noted, all fly crosses were conducted at 25°C and phenotypes are depicted in female progeny. Transgenic *dE2f1-dsRNA* (*dE2f1^{RNAi}*) flies were created using a system developed previously²⁷. SW480 (Antony Burgess), DLD1 (William Hahn), U2OS-*shRb* (Scott Lowe), and Saos2-*TR-E2F1* cells² were cultured in Dulbecco's modified Eagle's

medium supplemented with 10% fetal bovine serum. *Drosophila* Schneider line 2 (SL2) cells were maintained as previously described². Transient transfections were performed using Fugene-6 reagent (Roche), and in some cases with CellFectin (Invitrogen), according to the manufacturers instructions. For SW480 survival and DLD1 qPCR experiments, high efficiency (approximately 70%) gene transfer was accomplished by using Amaxa nucleofection according to the manufacturer's protocol (Program T-020; Kit-T and Kit-L, respectively). All *Drosophila* RNAi in SL2 cells was performed as described², using 50 µg double-stranded RNA (dsRNA) synthesized with T7 RiboMax (Promega) with all conditions normalized using *luciferase*-dsRNA. Luciferase reporter assays were performed as previously described for SL2 cells² and mammalian cells²⁸. MTT viability assay was performed as previously described². Western blot and immunohistochemical analysis was performed using standard techniques. Dissected third instar larval discs immunohistochemistry was performed using anti-dE2F1 antibody (Terry Orr-Weaver). Immunohistochemical staining of *Apc^{Min}* mouse tumors was performed as described¹⁹ using anti-Rb (Santa Cruz, sc-50) and β -catenin antibodies (BD Biosciences, 610054). ChIP and data analysis were carried out as previously described²⁹. Gel shift assays were performed as described³⁰. Detailed information on antibodies, fly stocks, and plasmids, as well as qPCR, GST-pulldown assay, co-immunoprecipitation, and IP-kinase assay conditions are all described in Supplementary Methods.

METHODS

Fly stocks, transgenes, and genetic crosses

The following stocks were used for these studies: *GMR-Gal4*, *ptc-Gal4*, *UAS-arm* (S2; 4783); *UAS-dTCF/pan* (4837); *UAS-dTCF Δ N/pan* (4784); *UAS-GSK3/sgg* (5361) (Bloomington Stock Center); *GMR-arm** (Y55; Mariann Bienz); *dCdk8^{K185}* (null) and *dCycC^{Y5}* (null) (Henri-Marc Bourbon); *GMR-wIR* (Richard Carthew); and *PCNA-GFP* (Bob Duronio). The *dE2f1^{RNAi}* primer sequence (sub-cloned inverted into the pWIZ vector) was 5'-TTATTTCAAACGCCCTACCG-3' and 5'-GAATTGCATCTGCAGTGAGC-3' (verified by sequencing). All transgenic fly embryo injections were performed by the CBRC Transgenic Fly Core. Approximately thirty different transgenic lines carrying one or multiple transgenes were balanced and recombined with different *Gal4* lines using standard *Drosophila* genetic methods. Since the *dE2f1^{RNAi}* phenotypes in both the *Drosophila* eye (*GMR-Gal4*, *UASdE2f1^{RNAi}* #10) and wing (*ptc-Gal4*, *UAS-dE2f1^{RNAi}* #3) could be modified by known factors that interact with the RB-E2F pathway (JYJ and NJD, unpublished observation), we designed and carried out a dominant modifier genetic screen. We screened ~6500 *piggyBac* transposon insertion lines of the Exelixis mutant collection (JYJ, AH and NJD, unpublished results) that was maintained at the Harvard *Drosophila* Collection (Spyros Artavanis-Tsakonas). A detailed description of the genetic screen will be presented elsewhere.

Cell Culture and Gene Transfer

Plasmids used include *pCMV-E2F1*, *pCMV-DP1*, *pCMV-E2F2*, *pCMV-E2F3*, *pCMV-E2F4*, *pCMV-E2F1 Δ X*, *pCMV-E2F1-121*, *pCMV-E2F1-143*, *pCMV-E2F1-144*, *pGST-E2F1*, *pGST-E2F4*, *pGST-DP1* (Kristian Helin); *pSFFV-p53* (Stanley Korsmeyer); *pCMV-neoBam*, *pCMV- β -gal4*, *pCMV-pRB*, *pE2F4B* (Fred Dick); *pCMV-DNDP1(Δ 103–126)* (Marie Classon); *8xSuperTop*, *8xSuperFop*, *pCMV-CDK8*, *pCMV-CDK8KD*, *pLKO-shCDK8-1*, *pLKO-shGFP* (William Hahn); *pCMV-E2F1/3 chimeras* (Joe Nevins); *pcDNA3-DNTCF1*, *pcDNA3-DNTCF4* (Hans Clevers); *pEVR-TCF1E* (Marian Waterman); *pGL3OT*, *pGL3OF* (Elizabeth Hay); *pEGFP-N1* (BD Biosciences); *pLPC-empty*, *pLPC-TAp73*, *pCneo- Δ 45- β -catenin*, *pCneo-S33Y- β -catenin*, *pCneo- β -catenin*, *LLP-shGFP*, and *LLP-shRb^{CD}* (targeting sequence GGTGTGTGCGAAATTGGATCA) (James Rocco). Inhibitors and

chemical reagents include LiCl GSK3 inhibitor (Fisher, 121–500), SB216763 GSK3 β inhibitor (Sigma, S3442), BOC-Aspartyl-FMK caspase inhibitor (Enzyme Systems Products, FK-011), MG101 (Sigma, A6185), and MG132 (Sigma, C2211).

Luciferase Reporter and EGFP viability assays

For pTopFLASH assays, Saos2 cells were transfected in 6-wells with 100–200 ng *pTopFLASH* reporter plus 100 ng S33Y- β -catenin expression construct, along with 100 ng *pCMV- β -gal* as normalization control following β -gal assay and, unless otherwise specified, luciferase assays were performed 48 hours after transfection (data is expressed as mean $\pm$ SD, N = 3). The S33Y- β -catenin construct was omitted for *pTopFLASH* assays in colorectal cancer cells. For EGFP co-transfection viability assay, 100 ng of *pEGFP-N1* was co-transfected along with the indicated plasmids. Whole cell lysates were prepared with GFP homogenizing buffer and assayed fluorometrically 48 hours after transfection (excitation/emission λ 488/511 nm). Tet-induced survival experiments were done under low serum (0.5%) conditions.

Western Analysis, Antibodies, and Immunohistochemistry

Other antibodies used include dE2F1 (polyclonal anti-rabbit, Carol Seum), E2F1 (Santa Cruz, sc-193), E2F4 (Santa Cruz, sc-1082), DP1 (Santa Cruz, sc-610), CRSP70 (Santa Cruz, sc-9426), c-myc (Santa Cruz, sc-40), TCF1 (Santa Cruz, sc-8589), TCF4 (Santa Cruz, sc-8632; Upstate, 05–511 for ChIP), Cyclin E (Santa Cruz, sc-247), Cyclin A (Santa Cruz, sc-596), pan-MAPK (BD Biosciences, 612641), β -catenin (Cell Signaling, 9562), CDK8 (Santa Cruz, sc-1521), pRB (Santa Cruz, sc-50), Arm (N27A1; Developmental Studies Hybridoma Bank), β 1-integrin (BD Biosciences, 610467), E-cadherin (BD Biosciences, 610181), N-cadherin (BD Biosciences, 610920), α -catenin (BD Biosciences 610193), FAK (BD Biosciences 610087), phospho-FAK Tyr397 (BD Biosciences, 611806), GSK3 (Cell Signaling, 9315), phospho-GSK3 (Upstate, 05–413), Src (Cell Signaling, 2108), phospho-Src Tyr416 (Cell Signaling, 2101), STAT3 (Cell Signaling, 9132), phospho-STAT3 Tyr705 (Cell Signaling, 9131), phospho-STAT3 Ser727 (Cell Signaling, 9134), STAT5 (Cell Signaling, 9310), phospho-STAT5 Tyr694 (Cell Signaling, 9356), SHC (BD Biosciences, 610081), anti-HA-epitope (Sigma, H6908), and GFP (Sigma, G1544).

Real-time quantitative PCR (qPCR)

Total RNA was prepared using RNeasy extraction Kit (Qiagen). Reverse transcription PCR (RT-PCR) was performed using Taq Man® Reverse Transcription (PE Applied Biosystems) according to manufacture specification. Real-time PCR was performed using an ABI prism 7900 HD Sequence Detection system. Relative mRNA level were determined using the SYBR Green I detection chemistry system (Applied Biosystems, Foster City, CA). Quantification was performed using the comparative C_T method as described in the manufacturer manual and *GAPDH* or *18S rRNA* was used as normalization control. All primers were designed with Primer Express 1.0 software (Applied Biosystems, Foster City, CA) following the manufacturer's suggested conditions.

Forward and reverse primer sequences include:

E2F1-f (CATCCCTCACCACAGATCCC), E2F1-r (AACAGCGGTTCTTGCTCCAG)

c-MYC-f (CGGATTCTCTGCTCTCCTCG), c-MYC-r
(CCACAGAAACAACATCGATTCTT)

AXIN1-f (ACAGCATCGTTGTGGCGTACT), AXIN1-r
(CACAGTCAAACGTCGCTCAC)

AXIN2-f (ATTCGGCCACTGTTTCAGACG), AXIN2-r
(GACAACCAACTCACTGGCCTG)

SIAH1-f (ATGGTCATAGGCGACGATTGAC), SIAH1-r
(AAGCTGTGCAATGCTGGTGTG)

CD44-f (CTGCAAGGCTTTCAATAGCACC), CD44-r
(CGTGCCCTTCTATGAACCCAT)

PPAR δ -f (CTTCCACTACGGTGTTCATGCA), PPAR δ -r
(GCACTTGTGCGGTTCTTCTTC)

CCNE1-f (TGCAGAGCTGTTGGATCTCTGTG), CCNE1-r
(GGCCGAAGCAGCAAGTATACC)

CDC25-f (TGAATACGAGGGAGGCCACATCAA), CDC25-r
(ACACGCTTGCCATCAGTAGGTACA)

TP73-f (CGTACTCCCCGCTCTTGAAG), TP73-r
(TCCGCTTTCTTGTAACAGGC)

GAPDH-f (CATGTTTCGTCATGGGTGTGAACCA), GAPDH-r
(GTGATGGCATGGACTGTGGTCAT)

Chromatin Immunoprecipitation

Chromatin extracts were incubated at 4°C overnight with antibodies specific for E2F1, CDK8, and anti-HA antibody as control. Immunocomplexes were recovered with protein A and G Sepharose beads. DNA was recovered and dissolved in 150 μ L of water. Real-time qPCR analysis was performed as described above.

Forward and reverse primer sequences include:

P107-f (GGCCAAGGACAGGTCTTTTCAG), P107-r
(AAAGACGCCAGAGATGCAGC)

CCNA1-f (CTGCTCAGTTTCCTTTGGTTTAC), CCNA1-r
(AAAGACGCCAGAGATGCAGC)

ACYLCOA-f (CCTTCATTGGGATCACCACG), ACYLCOA-r
(GGAGATGAGTACCAGCAGGTTG)

MYC-EBE-f (TCCGCCTGCGATGATTTATAC), MYC-EBE-r
(CAGAGTAAGAGAGCCGCATGAA)

MYC-TBE1-f (TCTCCCGTCTAGCACCTTTGA), MYC-TBE1-r
(CACGGAGTTCCCAATTTCTCAG)

MYC-TBE2-f (GCTCTCCAAGTATACGTGGCAA), MYC-TBE2-r
(TCAGAGCGTGGGATGTTAGTGTA)

GST-pulldown Assay

GST-fusion proteins were expressed in *E. coli* BL21 cells and purified from the lysates by glutathione sepharose beads (Pharmacia) with extensive washing. The amount bead-bound GST protein was determined by SDS-PAGE and Coomassie staining and normalized. The dE2F1 fragments, shown in Supplementary Fig. 7, were subcloned into *pGEX-2TKN* vector. To make Myc-tagged dE2F1 and HA-tagged dCDK8, full-length cDNAs were subcloned into *pcDNA4TO* or *pcDNA3HA*, respectively. Each construct was expressed in 293T cells and extracted in binding buffer (20 mM HEPES pH 7.6, 140 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM Benzamidine, 0.2 mM

phenylmethylsulfonyl fluoride (PMSF), and 1 µg/mL Aprotinin) with 0.5% NP-40. The whole cell extracts (WCE) were diluted 4-fold with binding buffer without detergent. S2 cell extracts were prepared similarly. WCE was applied to 50 µL of GST-fusion protein beads and incubated at 4°C for 3 hours. Beads were washed seven times with 1 mL wash buffer containing 50 mM Tris-HCl at pH 8.0, 250 mM KCl, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM DTT, 1 mM Benzamidine, 0.2 mM PMSF, and 1 µg/mL Aprotinin. The interacting proteins were then eluted with two bead volumes of binding buffer plus 0.3% sarkosyl at 4°C for 1 hour.

Co-immunoprecipitation

Saos2-*TR-E2F1* cells were collected and rinsed with 1×PBS after culture in the presence of 0.2 µg/mL of tetracycline for 12 hours. Cell pellets were re-suspended in IP buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1 mM EDTA, 10% Glycerol, 0.1% NP-40, 1 mM DTT, 0.2 mM PMSF, 1 mM Benzamidine, and 1 µg/mL Aprotinin). WCE was prepared by passing through 20(1/2)G needle for five times followed by incubation on ice for 15 minutes and centrifugation at 12,000 × g for 20 minutes. For immunoprecipitation, whole cell extracts were incubated with 30 µL of Protein-G beads pre-coated with antibodies against CDK8 (sc-1521), E2F1 (sc-193), control normal rabbit serum (IgG), or CRSP70 (goat anti-human CRSP70, Santa Cruz sc-9426). After rotating at 4°C for 2 hours, the beads were washed five times with 1 mL IP buffer. The antigen-associated proteins were eluted with 0.3% sarkosyl in IP buffer, resolved on 10% SDS-PAGE, and visualized using standard western analysis.

IP-kinase assay

CDK8 or control (CRSP70) proteins were immunoprecipitated from whole cell extracts of Saos2-*TR-E2F1* cells as described in the Co-IP section. The beads were washed twice with 1 mL IP buffer followed by two washes with 1 mL kinase assay buffer (50 mM Tris-HCl pH 7.4, 50 mM NaCl, 10 mM MgCl₂, 1 mM MnCl₂, 10% Glycerol, 0.2 mM PMSF, 5 mM DTT, and 1 µg/ml Aprotinin). For kinase assays, the resulting beads were incubated in 50 µL of kinase assay buffer containing 20 µCi of ³²P-γ-ATP at room temperature for 30 minutes. The reaction was terminated and the kinase-associated proteins were eluted with 0.3% sarkosyl in IP buffer. After diluted ten times with IP buffer, equal amount of the supernatant was applied to Protein-A beads pre-coated with antibodies against IgG (normal rabbit serum), E2F1 (sc-193), E2F4 (sc-1082), or DP1 (sc-610). The mixture was rocked at 4°C for 2 hours and the beads were washed four times with 1 mL with IP buffer. The samples were boiled, resolved in 12% SDS-PAGE, and then transferred to a PVDF membrane. The in-vitro-phosphorylated proteins were visualized by autoradiography.

Electrophoretic Mobility Gel Shift Assay

Briefly, 2 µg of nuclear extracts were incubated with ³²P-labeled double-stranded oligonucleotides containing E2F or TCF sites site (<10 pg) for 30 minutes at 4°C in binding buffer. For supershift experiment, antibodies were preincubated for 30 minutes. Samples were then loaded on a 4% polyacrylamide gel and visualized by autoradiography.

Double-strand oligonucleotide probe sequences include:

MYC-wtE2F (5'-GCTTCTCAGAGGCTTGCGGGAAAAAGAA)

MYC-mtE2F (5'-GCTTCTCAGAGGCTTGAGGGAAAAAGAA)

MYC-TBE1 (5'-GTCTAGCACCTTTGATTTCTCCCAAACCC)

MYC-TBE2 (5'-CTGGGACTCTTGATCAAAGCGCGGCCCTT)

TOP (5'-GCCCCCTTTGATCTTACCCCTTTGATCT)

Canonical wtE2F (5'-ATTTAAGTTTCGCGCCCTTTCTCAAATTT)

Canonical mtE2F (5'-ATTTAAGTTTCGATCCCTTTCTCAAATTT)

Supplementary Material

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Figure 1. Functional antagonism between E2F1 and β -catenin/TCF-signaling

(a) Normal *Drosophila* wing of *Act88F-Gal4/+* genotype. A *dE2F1*-induced apoptotic wing phenotype (b) is strongly suppressed by co-expression of *arm* (d) or *pan/dTCF* (e), phenocopied by expression of dominant-negative *dTCF* (*dTCF Δ N*) (f), and strongly synergizes with *sgg/GSK3* co-expression (h). Expression of *arm* (c), *sgg* (g), or *pan* (not shown) alone does not induce a wing phenotype. (i-l) *GMR*-mediated eye-specific expression of *dE2F1/dDP* strongly suppresses a rough eye phenotype induced by activated-*arm* (*arm**; *S44Y*) expression. All phenotypes were compared in female progeny from F1 crosses conducted at 25°C versus control (*w¹¹¹⁸* background). (m) E2F1 expression activates a canonical pE2F4B-luciferase reporter while abrogating the S33Y- β -catenin-mediated activation of pTopFLASH. (n) Inhibition of pTopFLASH activity is specific to E2F1. (o) E2F1 dominantly inhibits pTopFLASH activation by p73. All data is expressed as mean $\pm$ s.d. (n = 3) of normalized relative light units (NRLU) of luciferase.

Figure 2. E2F1 abrogates Wnt signaling by modulating β -catenin target gene expression and inducing the GSK3-independent degradation of β -catenin

(a) In Saos2-TR-E2F1 cells, E2F1 represses c-myc levels without affecting the levels of TCF1 or TCF4. (b–d) E2F1 modulates the expression of endogenous Wnt target genes (qPCR analysis after Tet-induced E2F1 expression at 24 hours). (e) The kinetics of E2F1-induced *AXIN2* and *SIAH1* expression mirrors the E2F1 activation of *CCNE1/Cyclin E* and *CCNA1/Cyclin A*, and precedes E2F1-induced apoptosis. (f) E2F1 represses the expression of Wnt targets in DLD1 colorectal cancer cells. Levels of mRNA were normalized to GAPDH and the effect of E2F1 is depicted as the ratio between samples after *pCMV-empty* or *pCMV-E2F1* (1 μ g each) expression. (g, h) E2F1 induced β -catenin degradation (control expression from the same lysates shown in Supplementary Figure 4f) is both GSK3- and caspase-independent. Saos2 cells were treated with control (DMSO), GSK3 inhibitors (20 μ M SB216763 and 5 mM LiCl), or the caspase inhibitor peptide BOC-aspartyl-FMK (BAF; 100 μ M) with or without Tet-induction of E2F1 (Western blot). (i) Co-expression of Bcl-2 (25 ng), pRb (10–25 ng), stabilized tumor-derived β -catenin mutants (10–25 ng), or the GSK3 β inhibitor SB216763 (15 μ M), is sufficient to partially-rescue E2F1-induced apoptosis. Cell death was depicted as percent inhibition of EGFP loss at 48 hours after transfection with *pCMV-E2F1* or *pCMV-empty* (100 ng each) along with *EGFP* expression construct. All data is expressed as mean $\pm$ s.d. (n = 3).

Figure 3. pRB inactivation abrogates β -catenin/TCF-dependent transcription

(a–b) High levels of pRB and β -catenin colocalize within the tumor epithelium of an *Apc^{Min}* colonic tumor. (c) Endogenous pRB was depleted for 6 days in U2OS-*shRb* cells (containing DOX-inducible short-hairpin-*Rb* and *GFP* transgenes) (d) pRB depletion activates E2F and represses TCF activity. Basal and activated E2F and TCF activity was determined by transfection of their respective luciferase reporter plasmids (for 24 hours) in the absence (basal) or presence (activated) of E2F1 or S33Y- β -catenin expression constructs. (e) In SW480 colorectal cancer cells, E2F1 expression is sufficient to activate E2F activity and repress endogenous β -catenin activity in a dose-dependent manner. (f) Expression of short-hairpin pRB (400 ng) is sufficient to repress pTopFLASH activity in three different

colorectal cancer cell lines (SW480, DLD1, and HCT116). (g) pRB inactivation reduces clonogenic survival of SW480 cells and is partially rescued by *S33Y- β -catenin/TCF1E*, but not *Bcl-2* expression. SW480 cells were Amaxa nucleoporated with either control *LLP-GFP* or *-Rb* silencing constructs (1 μ g each) with or without *S33Y- β -catenin* (250 ng), *TCF1E* (250 ng), or *Bcl-2* (500 ng) expression constructs, and survival was determined at day 5 by MTT assay. All data is expressed as mean $\pm$ s.d. (n = 3; *p < 0.015 by T-test).

Figure 4. CDK8 antagonizes E2F1 activity

(a–c) The *dCdk8^{c01804}* mutant dominantly suppresses a rough eye phenotype caused by the eye-specific expression of a *dE2F1^{RNAi}* transgene. (d) The expression of the dE2F1 target genes, *PCNA* and *MCM5* are upregulated in *dCdk8* (null or hypomorphic alleles) or *dCycC* null mutant *Drosophila* larvae, while the expression of the dE2F2 target gene *vasa* is unaffected. (e) dCDK8 physically interacts with dE2F1 by GST-pulldown assay. (f) Co-immunoprecipitation of human E2F1 and CDK8 from Saos2 whole-cell extracts. As control, E2F1 does not associate with the CRSP70 small-Mediator subunit. (g) CDK8 binds to and specifically phosphorylates E2F1. Kinase-assay was performed following CDK8 or CRSP70 (as control) immunoprecipitation from Saos2 cells. E2F1, E2F4, or DP1 were re-

immunoprecipitated and resolved in 12% SDS-PAGE. **(h)** The expression of wild-type CDK8, but not kinase-dead, abrogates the inhibitory effects of E2F1 on β -catenin/TCF-dependent transcription. pTopFLASH assays were determined at 48 hours in co-transfection experiments with 200 ng CDK8 or CDK8KD in Saos2 cells. **(i)** Expression of short-hairpin pRB (400 ng) and CDK8KD (300 ng) co-operatively repress pTopFLASH activity in SW480 colorectal cancer cells. All data is expressed as mean $\pm$ s.d. (n = 3).