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## RNF4-Dependent Oncogene Activation by Protein Stabilization

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### SUMMARY

Ubiquitylation regulates signaling pathways critical for cancer development and, in many cases, targets proteins for degradation. Here, we report that ubiquitylation by RNF4 stabilizes otherwise short-lived oncogenic transcription factors, including  $\beta$ -catenin, Myc, c-Jun, and the Notch intracellular-domain (N-ICD) protein. RNF4 enhances the transcriptional activity of these factors,

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### SUPPLEMENTAL INFORMATION

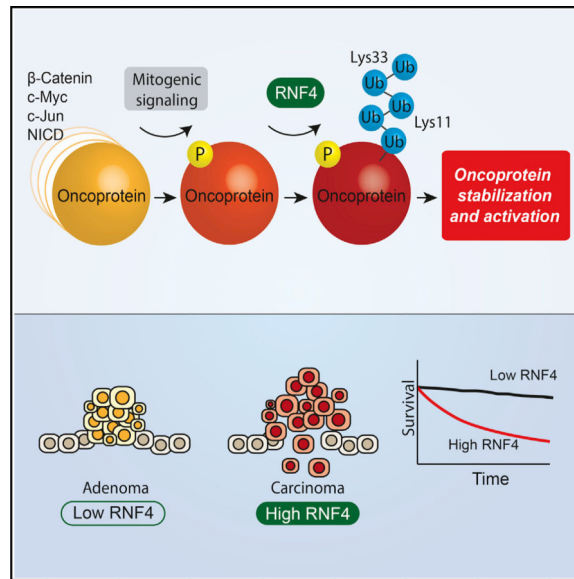
Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at <http://dx.doi.org/10.1016/j.celrep.2016.08.024>.

### AUTHOR CONTRIBUTIONS

M.A. discovered that RNF4 stabilizes short-lived oncogenes. J.J.T. discovered that phosphorylation, but not SUMOylation, is required for recognition, and the motifs within RNF4 mediating the interaction. J.J.T. determined the nature of atypical ubiquitylation and, together with J.H., that RNF4 potentiate tumors phenotypes. J.H. discovered that RNF4 is required for endogenous Wnt activation and endogenous Myc stability. M.A. and J.H. developed RNF4 targeting and overexpression systems. All co-first authors designed and performed experiments, and analyzed data. R.N. and Y.Z. performed cancer cell and pathological analyses, respectively. J.S.T.-A., A.P.L.B., and M.I. performed cellular and transcriptional analyses. D.B. generated a critical Myc expression vector. A.O. designed and performed stability experiments. All authors designed experiments and analyzed data. J.J.T., M.A., J.H., W.B., A.L.S., and A.O. wrote the manuscript.

as well as Wnt- and Notch-dependent gene expression. While RNF4 is a SUMO-targeted ubiquitin ligase, protein stabilization requires the substrate's phosphorylation, rather than SUMOylation, and binding to RNF4's arginine-rich motif domain. Stabilization also involves generation of unusual polyubiquitin chains and docking of RNF4 to chromatin. Biologically, RNF4 enhances the tumor phenotype and is essential for cancer cell survival. High levels of *RNF4* mRNA correlate with poor survival of a subgroup of breast cancer patients, and RNF4 protein levels are elevated in 30% of human colon adenocarcinomas. Thus, RNF4-dependent ubiquitylation translates transient phosphorylation signal(s) into long-term protein stabilization, resulting in enhanced oncoprotein activation.

## Graphical Abstract



## INTRODUCTION

Post-transcriptional modifications of nuclear oncoproteins play important roles in cancer. Many oncogenic transcription factors are phosphorylated by mitogenic signaling pathways that enhance their activity. In addition, modifications by ubiquitin and ubiquitin-like proteins, such as small ubiquitin-like modifier (SUMO), regulate critical signaling pathways, as well as transcription factors (Bassermann et al., 2014; Flotho and Melchior, 2013; Swatek and Komander, 2016). In many cases, phosphorylation primes the protein for ubiquitylation, which often results in its degradation and loss of oncogenic activity (López-Otín and Hunter, 2010). Understanding how post-transcriptional modifications like phosphorylation, ubiquitylation, and SUMOylation are connected and act in concert to regulate transcriptional activity is an area of active research.

One such connector is the RING ubiquitin ligase RNF4, a SUMO-targeted ubiquitin ligase (STUbL) (Sriramachandran and Dohmen, 2014). STUbLs are conserved in evolution and detected in yeast, flies, and mammals, and two members RNF4 and RNF111 exist in vertebrates. STUbLs directly connect the SUMO and the ubiquitin pathways: STUbLs bind

to SUMOylated chain(s) of target proteins via their SUMO-interacting motifs (SIMs) then ubiquitylate these proteins, which often results in their subsequent proteasomal degradation. Moreover, STUbL-mediated ubiquitylation does not only target proteins for degradation. For example, STUbL-mediated ubiquitylation affects the affinity of protein-protein interactions, which, in turn, affects subcellular localization, as well as enhances transcriptional activation (Fryrear et al., 2012; Poukka et al., 2000; Hu et al., 2010). Previously, we showed that Dgrn, the sole *Drosophila* STUbL protein, inactivates the SUMOylated co-repressor Groucho/Transducing-like enhancer of split (Gro/TLE) and inhibits Gro-dependent gene repression in vivo (Abed et al., 2011).

Gro/TLE proteins inhibit signal-dependent transcription (i.e., Wnt/ $\beta$ -catenin signaling) in development and cancer (Cinnamon and Paroush, 2008). The key co-activator of the Wnt pathway,  $\beta$ -catenin, is usually unstable, and phosphorylation by GSK-3 targets  $\beta$ -catenin for proteasomal degradation. Following Wnt pathway activation, cytoplasmic  $\beta$ -catenin is stabilized and translocates to the nucleus. Subsequently, nuclear  $\beta$ -catenin displaces the co-repressor Gro/TLE from TCF4, which results in activation of TCF4/ $\beta$ -catenin target genes, including Axin2 and c-Myc (Figure 1A; Holland et al., 2013; Clevers et al., 2014). Moreover, *RNF4* is a direct c-Myc target, and its expression is dependent on Wnt/ $\beta$ -catenin activity (Dutta-Simmons et al., 2009; Reymann and Borlak, 2008).

Our observations that STUbLs antagonize Gro/TLE (Abed et al., 2011; Barry et al., 2011) prompted us to determine whether RNF4 plays a role in Wnt signaling. Here, we show that RNF4 enhances Wnt signaling by antagonizing TLE1 and concomitantly stabilizing  $\beta$ -catenin protein. In addition, RNF4 stabilizes other phosphorylated nuclear oncoproteins, including c-Myc, NICD, c-Jun, and PGC1 $\alpha$ . RNF4-mediated oncogene stabilization requires binding of RNF4 to its substrates in their phosphorylated forms. This activity of RNF4 is highly relevant to cancer; RNF4 is a positive feedback enhancer of Wnt- and Notch-dependent gene expression. It is essential for cancer cell survival, and its expression potentiates tumorigenic properties of cancer cells. High *RNF4* mRNA levels correlate with poor survival of a subgroup of breast cancer patients, and RNF4 protein levels were elevated in 30% of human biopsies of colon adenocarcinoma, but not in biopsies of normal colon or adenomas, suggesting a role for RNF4 in tumor progression.

## RESULTS

### RNF4 Enhances Wnt/ $\beta$ -Catenin-Dependent Transcriptional Activity

In *Drosophila*, the RNF4 ortholog Degrin (Dgrn) antagonizes Groucho-dependent transcriptional repression (Abed et al., 2011). Groucho, and its vertebrate ortholog TLE1, are potent inhibitors of Wnt-dependent transcriptional activation (Levanon et al., 1998). Therefore, we tested whether RNF4 relieves TLE1-mediated repression of the Wnt luciferase reporter TOPFLASH in HEK293T cells (TOP; Figure 1A; Levanon et al., 1998; van de Wetering et al., 1991). Transfected TLE1 suppressed  $\beta$ -catenin-induced activation of the TOP reporter. Co-expression of RNF4, but not the mutant RNF4<sup>C159A</sup> which lacks ubiquitylation activity, alleviated this repression and enhanced transcriptional activation (Figure 1B). RNF4 enhanced both TCF/ $\beta$ -catenin and LiCl-induced transcriptional activation, and this was observed in the presence and absence of transfected (exogenous)

TLE1 (Figures 1C and S1A). RNF4 also enhanced transcriptional activation induced by  $\beta$ -catenin<sup>Y33A</sup>, a constitutively active oncogenic form of  $\beta$ -catenin (Figure S1B; Morin et al., 1997). This potentiation of transcriptional activation by RNF4 depends on the binding of the TCF/ $\beta$ -catenin activation complex to DNA, as RNF4 had no impact on a reporter that lacks TCF binding sites (FOPFLASH; FOP), suggesting that RNF4 does not affect the general transcriptional machinery in the absence of the activation complexes (Figure S1C).

Next, we determined whether endogenous RNF4 is required for the transcriptional activation of Wnt3a/ $\beta$ -catenin target genes in various cancer cell lines. Constitutive RNF4 small hairpin RNAs (shRNAs) (shRNF4-C1, shRNF4-C2), but not control (scshRNA), efficiently reduced *RNF4* mRNA and endogenous protein levels in MDA-1833 breast cancer cells and HCT116 colon carcinoma cells (Figures 1D, S1D, and S1E). We analyzed the expression of endogenous *Axin2* mRNA, a direct Wnt3a/ $\beta$ -catenin target and a transcriptional readout for the pathway (Heuberger et al., 2014). In HCT116 colon carcinoma cells, RNF4 knockdown reduced *Axin2* mRNA expression four hours after Wnt3a treatment, as well as in the basal state (Figure 1E). We observed a similar effect in MDA-1833 breast cancer cells where we activated  $\beta$ -catenin-dependent transcription by addition of the GSK-3 inhibitor Chir99021 (Figure 1F). RNF4 knockdown in HEK293T cells, which express low endogenous RNF4 levels, resulted in a modest, yet statistically significant, decrease in TOP-luciferase activity (Figures 1D and S1F).

### RNF4 Stabilizes and Binds to Phosphorylated $\beta$ -Catenin and c-Myc

While monitoring protein levels in the cells used in the reporter assays above, we noticed that RNF4-expressing cells had higher levels of  $\beta$ -catenin and its target c-Myc. We found that RNF4 expression increased the levels of  $\beta$ -catenin and endogenous c-Myc proteins, and this required RNF4's catalytic activity (Figures 1B and 1C). RNF4-dependent increase in endogenous c-Myc protein was also observed in cells that do not overexpress  $\beta$ -catenin (Figure 2A). It was also detected when c-Myc expression was driven by an exogenous CMV promoter, (i.e., not dependent on the endogenous c-Myc promoter; Figure 2B). This function is conserved in evolution and co-expression of *Drosophila melanogaster*, *Rattus norvegicus*, or *Homo sapiens* RNF4 orthologs in HEK293T cells, all significantly elevated exogenous c-Myc protein levels (Figure 2B). Moreover, reduction of RNF4 by two independent Doxycycline (Dox)-induced shRNF4s, but not control shRNA (Renilla), caused a decrease in the protein level of endogenous c-Myc in MDA-MB-231 cells (Figures 2C and S1G). Elevated c-Myc level was due to protein stabilization, as RNF4 stabilized HA-c-Myc in cycloheximide and <sup>35</sup>S-methionine-labeling pulse-chase experiments (Figures 2D and 2E). RNF4 also stabilized other Myc protein family members such as N-Myc and L-Myc (Figure S2A; data not shown).

c-Myc protein activity and stability are regulated by phosphorylation: mitogenic signals mediated by multiple kinases such as Erk/Mapk or Cdk2 phosphorylate Ser62 of c-Myc, which initially stabilizes c-Myc and enhances its transcriptional activity (Sears et al., 2000). Ser62 phosphorylation also primes c-Myc for GSK3-dependent phosphorylation of Thr58 that subsequently marks it for degradation (Welcker et al., 2004; Farrell and Sears, 2014). We observed that RNF4 stabilized p-Ser62 c-Myc in a pronounced manner, using an

antibody specific for singly phosphorylated p-Ser62 (Figure 2F). Similarly, reduction of RNF4 resulted in a significant decrease in endogenous p-Ser62-c-Myc protein level (Figure 2G). RNF4-dependent c-Myc stabilization was abolished by mutation of S62A, but not T58A, in both steady-state and dynamic experiments (Figures 2H and 2I). Likewise, chemical inhibition of kinases known to phosphorylate Ser62 impaired the ability of RNF4 to stabilize c-Myc (Figure 2J).

In agreement, we observed that glutathione S-transferase (GST)-fused RNF4 binds to phosphorylated <sup>35</sup>S-Met-labeled c-Myc in vitro. This binding was greatly reduced when we used the non-phosphorylatable <sup>35</sup>S-Met-c-Myc<sup>S62A</sup> mutant, which was also not stabilized by RNF4 (Figures 2H, 2I, and 3A). Likewise, pre-treatment of c-Myc with calf intestinal phosphatase (CIP) abolished RNF4-binding (Figures 3B and S2B). Furthermore, c-Myc was highly ubiquitylated by RNF4 in cells and in a cell-free ubiquitylation system (Figures 3C and S3A). In contrast, the c-Myc<sup>S62A</sup> mutant was poorly ubiquitylated (Figure 3C, upper). Moreover, we found that RNF4 stabilizes Ser45 phosphorylated  $\beta$ -catenin. Similar to the case of Ser62-c-Myc, phosphorylation of Ser45 within  $\beta$ -catenin enhances its activity (Maher et al., 2010), but also primes  $\beta$ -catenin for GSK-dependent phosphorylation and degradation (Amit et al., 2002). We found that RNF4 is required for maintaining endogenous  $\beta$ -catenin protein level, and that phosphorylation of  $\beta$ -catenin Ser45 is required for stabilization, binding, and ubiquitylation of  $\beta$ -catenin by RNF4 (Figures 3D–3G, S2C, and S2D). Taken together, these data suggest that RNF4 binds to p-Ser62-c-Myc and p-Ser45- $\beta$ -catenin, and targets each for ubiquitylation, which in turn leads to their stabilization.

### **RNF4's ARM Domain and Atypical Polyubiquitin Chains Are Required for c-Myc and $\beta$ -Catenin Stabilization**

To understand how RNF4 interacts directly with its substrates, we defined the structural motifs within RNF4 that recognize phosphorylated c-Myc and  $\beta$ -catenin. RNF4's short arginine-rich motif (ARM; aa residues 72–82), along with its SIM domains, were recently shown to recognize the phosphorylated and SUMOylated KAP1 protein (Figure 4A; Kuo et al., 2014). While wild-type RNF4 stabilized c-Myc and  $\beta$ -catenin, the ARM deletion mutant, RNF4<sup>ARM</sup>, failed to bind, ubiquitylate, or stabilize these substrates (Figures 4B–4D, S2E, S4A, and S4B). Similarly, a mutation in RNF4's nucleosome-targeting region (NTR; RNF4<sup>K179D</sup>), which mediates its interaction with nucleosomes, failed to stabilize c-Myc or  $\beta$ -catenin (Figures 4A, 4C, and 4D; Grocock et al., 2014). In addition, both the ARM domain and K179 were required for RNF4-dependent enhancement of  $\beta$ -catenin and c-Myc-dependent transcriptional activation (Figures 4C and 4D). In contrast, the SIM domains were not required for binding to c-Myc (Figure S2F). Furthermore, expression of the RNF4 SIM domain mutant, RNF4<sup>SIM</sup>, which cannot bind to SUMOylated proteins, enhanced  $\beta$ -catenin and c-Myc-dependent transcriptional activation albeit to a more modest extent than RNF4 (Figures 1C, 4C, and 4D). These results show that RNF4's ARM domain is required for binding to the phosphorylated substrates, and suggest that protein stabilization occurs in the nucleus in the vicinity of or on chromatin.

Next, we asked how RNF4 ligase activity results in protein stabilization. We hypothesized that RNF4 ligase activity may inhibit substrate ubiquitylation and subsequent degradation, or

is required for catalyzing polyubiquitin chains that are not involved in targeting proteins for degradation. Indeed, high molecular weight endogenous and exogenous c-Myc-Ubiquitin conjugates were readily observed when ubiquitylation was monitored in HEK293T cells expressing RNF4, but not RNF4<sup>C159A</sup> mutant protein (even in the absence of proteasomal inhibitors; Figures 4E and 4F). Therefore, we assessed whether stabilization requires the catalysis of polyubiquitin chains by using a mutant ubiquitin that acts as a “chain terminator”. In this mutant ubiquitin (UbK0) all the internal Lys residues were replaced by Arg (Ziv et al., 2011). Herein RNF4 was unable to stabilize and ubiquitylate c-Myc in HA-UbK0-expressing cells (Figures 4G, 4H, 5A, S4C, and S4D). Moreover, we found that RNF4-dependent stabilization of pSer62-c-Myc and p-Ser45  $\beta$ -catenin involves the catalysis of unusual ubiquitin chains with internal linkage of K11, K33 of ubiquitin, but not K6, K27, K29, K48, or K63, (Figure 4G, quantitated in Figures 4H, 5A, S4C, and S4D). Remarkably, using ubiquitin mutants that each contain only a single Lys residue (K\*), we found that only co-expression of K11\* and K33\* was sufficient for stabilization and ubiquitylation of both c-Myc (Figures 4I and 5B), and  $\beta$ -catenin (Figures 4J and 5C). These observations fit well with our report that the *Drosophila* ortholog of RNF4, Dgrn, catalyzes mixed polyubiquitin chains (Abed et al., 2011).

The stabilizing effect of RNF4 may also be a result of compromising the machineries involved in the physiological degradation of c-Myc and  $\beta$ -catenin, which are targeted for proteasomal degradation by the SCF-ubiquitin ligase complexes SCF<sup>Fbw7</sup> and SCF <sup>$\beta$ TRCP</sup>, respectively (Welcker et al., 2004; Winston et al., 1999). We asked whether RNF4 compromises the activity of these ligases. Transfection of SCF<sup>Fbw7</sup> in HEK293T cells decreased c-Myc protein levels 3-fold (Figure 5D). Co-expression of RNF4 and SCF<sup>Fbw7</sup> also reduced c-Myc levels 3-fold compared to expression of RNF4 alone. Thus, RNF4 does not interfere with SCF<sup>Fbw7</sup>-dependent c-Myc degradation. Similarly, an established assay for SCF <sup>$\beta$ TRCP</sup> ligase activity is the TNF $\alpha$ -induced degradation of the cytoplasmic inhibitor of NF- $\kappa$ B, p-I $\kappa$ B (Yaron et al., 1998). RNF4 neither polyubiquitylated nor stabilized I $\kappa$ B $\alpha$ , and had no effect on TNF $\alpha$ -induced NF- $\kappa$ B transcription activation (Figures S3A–S3C). We also found that RNF4 does not target other components of the SCF complex for degradation, and that limiting E2 levels were not the cause of RNF4-dependent stabilization. Moreover, proteasome activity was identical in RNF4 or control expressing cells (Figures S3D–G). Thus, the stabilizing effect of RNF4 is direct, and is not due to an impact on the machinery that mediates the turnover of these proteins.

Interestingly, RNF4 stabilizes other transcription factors that are phosphorylated by mitogenic kinases and degraded by SCF<sup>Fbw7</sup>. These include, PGC1 $\alpha$  (peroxisome proliferator-activated receptor gamma co-activator 1 $\alpha$ ), c-Jun, and notch intra-cellular domain (NICD) protein, which were all stabilized and ubiquitylated by exogenous RNF4, but not RNF4-mutants (Figures 5E, 5F, 6A, 6C, S2G, S4E, and S4F). For example, NICD, the cleaved intracellular domain of Notch, acts as transcriptional co-activator for Notch targets (Kopan, 2012). Flag-NICD protein levels were increased following co-expression of RNF4 in MK4 cells (Figure 6A). These cells express endogenous Notch receptor, and can be activated by Delta to enhance expression of a Notch reporter. We observed that RNF4, but not RNF4<sup>C159A</sup>, enhanced Delta-dependent transcriptional activation of the reporter,



whereas the basal activity of the reporter was not affected (Figure 6B). These results indicate that NICD generated by ligand-dependent Notch cleavage is the target of RNF4.

### Protein Stabilization by RNF4 Is SUMOylation Independent

RNF4 recognizes SUMOylated substrates by its SIM motifs. However, in yeast the recognition of the non-SUMOylated protein, MAT $\alpha$  repressor, was described (Xie et al., 2010). Therefore, we determined whether RNF4-dependent stabilization require SUMOylation. We used a breast cancer cell line that expresses shRNA inactivating SAE2, a subunit of the SUMO-activating enzyme E1, in a doxycycline (Dox)-inducible manner inhibiting de-novo SUMOylation (MDA-MB-231<sup>DoxSAE2shRNA</sup> cells; Figure 6D) (Kessler et al., 2012). Remarkably, addition of Dox decreased SAE2 levels, and reduced de-novo SUMOylation, but did not affect RNF4-dependent stabilization of c-Myc,  $\beta$ -catenin, NICD, and c-Jun at steady state or c-Myc in pulse-chase experiments (Figures 6D–6G, S5A, and S5B). While SAE2 knockdown reduced the basal level of  $\beta$ -catenin (Figure 6E), the ability of RNF4 to stabilize  $\beta$ -catenin, and enhance  $\beta$ -catenin-dependent transcriptional activation was similar in the presence or absence of SAE2 (Figures 6E and S5C). Nevertheless, and as expected, in the presence of functional SAE2 RNF4 expression resulted in reduction in the cellular pool of poly-SUMOylated proteins (Figure S5D). Moreover, we tested whether expression of RNF4 in this context affects the stability of the endogenous proteins studied above. We infected MDA-MB-231<sup>DoxSAE2shRNA</sup> cells with lenti-viral vectors coding for Dox-induced expression of RNF4. Thus, following Dox addition these cells inactivate SAE2 and concomitantly express RNF4 (Figure 6H). We found that Dox addition resulted in higher levels of all these endogenous proteins including p-c-Myc, and p-c-Jun. Thus, RNF4-dependent protein stabilization and enhancement of transcriptional activation are independent of de-novo covalent SUMOylation.

### RNF4 Is Essential for Cancer Cells and Potentiates Tumor Cell Properties, and Its Levels Are Elevated in Distinct Subsets of Human Cancers

Since RNF4 stabilizes oncoproteins and potentiates their activity, we examined the effects of RNF4 inhibition and expression on cancer cell properties. Expression of constitutive shRNF4 inhibited proliferation of MDA-MB-231 cells, a triple-negative breast cancer cell line that is Myc-dependent (Figure 7A; Kessler et al., 2012). RNF4 knockdown also resulted in extensive cell death: 47% apoptotic cells were observed in RNF4-targeted cells compared to 6.8% in control cells (Figures S6A–D). Likewise, downregulation of RNF4 inhibited colony formation of these cells in soft agar, and similar results were observed in melanoma and osteosarcoma cells (Figures 7B and 7C; data not shown). These effects were not augmented when SUMOylation was co-inhibited (i.e., conditional SAE2 knockdown). Furthermore, downregulation of SAE2 alone did not result in cell death or impaired colony formation (Figures 7B and S6B). Remarkably, expression of c-Myc partially restored colony growth in soft agar of RNF4-knockdown cells (Figures 7B and 7C). These data fit well with our observation that RNF4 is required to maintain the level of c-Myc protein in MDA-MB231 cells (Figure 2C), and enhances its transcriptional activity (Figures 4D and S2H), suggesting that the essential role of RNF4 in these cells is mediated, in part, by its effect on c-Myc.

Moreover, RNF4 potentiates the tumorous properties of cancer cells. Dox-induced expression of RNF4 but not RNF4<sup>ARM</sup> or RNF4<sup>C159A</sup> enhanced sphere formation of MCF7 breast cancer cells that are less aggressive cancer cells in comparison with MDA-MB231 (Figures 7D). Remarkably, we observed that high levels of *RNF4* mRNA correlate with poorer survival in a cohort of systemically untreated “type A,” but not “type B,” luminal estrogen positive breast cancer patients (Figure 7E; data not shown).

The ability of RNF4 to enhance tumorigenesis in culture was not limited to breast cancer cells but was also observed in SW480 colon cancer cells. Conditional expression of RNF4 but not RNF4<sup>ARM</sup> or RNF4<sup>C159A</sup> in SW480 cells increased the number and size of spheres (Figures 7F, S6E–S6H', and S6I). Since RNF4 enhances sphere formation of colon cancer cells we determined whether RNF4 protein is overexpressed in human-derived specimens of intestinal tumorigenesis. We analyzed 99 patient-derived colon biopsies of which 33 correspond to normal mucosa, 32 to benign adenomas, 1 to a carcinoma in situ, and 33 to malignant adenocarcinomas (see examples in Figures 7G–7J'). Elevated RNF4 protein levels were only observed in 1 out of 65 benign or normal colon biopsies. However, 29% (9/34) of adenocarcinomas exhibited significantly elevated RNF4 protein levels ( $p < 0.001$ ). Interestingly, in the single case of an in situ carcinoma the elevated RNF4 protein level was confined to the malignant area and not found in the benign tissue (Figure 7I and 7I'). Thus, elevated RNF4 levels in a subset of malignant tumors suggest that RNF4 overexpression may define specific sub-groups of human breast and colon cancer.

## DISCUSSION

Modifications by ubiquitin and ubiquitin-like proteins are intimately linked to tumorigenesis (Popovic et al., 2014). In many cases ubiquitylation targets oncogenes for proteasomal degradation (Ciechanover et al., 1991). In contrast, RNF4-dependent ubiquitylation stabilizes a subgroup of oncogenic transcription factors. RNF4 directly recognizes and stabilizes its targets, and phosphorylation, but not de-novo-SUMOylation of these targets is a prerequisite for substrate recognition and stabilization. While phosphorylation of proteins is often transient (Hunter, 1995), RNF4-dependent stabilization of  $\beta$ -catenin, c-Myc, and NICD persists for hours. Hence, RNF4 translates short-term phosphorylation signals into long-lasting effects, augmenting the transcriptional output of Wnt and Notch pathways. This prolonged effect establishes a feed-forward mechanism that enhances oncogenic activity (Figures 6I and 6J). In accordance, RNF4 enhances cancer cell survival, and RNF4 levels are elevated in a subset of human colorectal and breast tumors correlating with poor outcome for the latter.

### Mechanisms of RNF4-Dependent Oncogene Stabilization

STUbL proteins like RNF4 recognize SUMOylated substrates (Sriramachandran and Dohmen, 2014). Remarkably, we find that RNF4 also binds and stabilizes its substrates in a manner that depends on phosphorylation but not SUMOylation. RNF4 substrates are nuclear transcriptional regulators that enhance G1-S transition and promote proliferation and tumorigenesis. They are activated by phosphorylation via kinases such as Mapk, Cdk2, p38, or CKI. Yet, the underlining mechanisms involved had not been fully identified. These



phosphorylations are essential for RNF4 binding, ubiquitylation and stabilization. Another example in which RNF4 recognizes a phosphorylated protein is KAP1 (Kuo et al., 2014). Yet KAP1 is recognized when it is phosphorylated and SUMOylated via both the ARM and SIM domains leading to its degradation. Thus, recognition by ARM alone and the dual recognition by both the ARM and SIM motifs results in ubiquitylation, but nonetheless have opposite outcomes for protein stability.

The question arises as to how these opposite outcomes of protein stability are achieved. We found that RNF4-dependent protein stabilization of c-Myc and  $\beta$ -catenin requires the catalysis of polyubiquitin chains linked at position UbK11 and UbK33, which are associated with immunity, cell-cycle progression and protein trafficking, (Iwai, 2014, Wickliffe et al., 2011, Yuan et al., 2014). However, in RNF4-dependent degradation of SUMOylated proteins, mixed SUMO-ubiquitin chains, as well as UbK48-linked chains, are formed. This ability to generate chains with different types of internal linkage may stem from distinct interactions of RNF4 with several E2 enzymes (Häkli et al., 2004; Guzzo et al., 2012). We suggest that the internal linkage of the ubiquitin chain may well determine whether RNF4-dependent ubiquitylation promotes protein degradation or stabilization.

### Regulation of Wnt Signaling and c-Myc by Ubiquitylation and SUMOylation

Ubiquitylation is known to regulate Wnt signaling beyond the degradation of  $\beta$ -catenin by SCF $^{\beta}$ TRCP. The ubiquitin ligases RNF43, and RNF14 modulate Wnt pathway activity but do not alter  $\beta$ -catenin stability or bind to  $\beta$ -catenin directly (Koo et al., 2012; Wu et al., 2013). A third ligase, EDD, stabilizes  $\beta$ -catenin but requires phosphorylation of Ser33 of  $\beta$ -catenin; the oncogenic  $\beta$ -catenin<sup>S33A</sup> mutant is unaffected by EDD (Hay-Koren et al., 2011). However, RNF4 enhanced  $\beta$ -catenin<sup>S33A</sup> activity, indicating that EDD and RNF4 target different pools of  $\beta$ -catenin modified by distinct mechanisms. Moreover, the observations that RNF4 enhances the activity of cancerous-related  $\beta$ -catenin mutants may offer a strategy to target cancers where the degradation of  $\beta$ -catenin is compromised.

Similar to ubiquitylation, SUMOylation regulates the Wnt pathway. We show here that targeting the SUMO E1 subunit SAE2 reduced  $\beta$ -catenin protein level and impaired  $\beta$ -catenin transcriptional activity, consistent with a previous report (Choi et al., 2011). However, RNF4-dependent protein stabilization as well as its transcriptional and biological activities were not affected by targeting SAE2 and inhibition of SUMOylation. SUMOylation was also reported to target c-Myc for degradation, and to maintain a c-Myc signature in MDA-MB-231 cells overexpressing MycER (González-Prieto et al., 2015; Kessler et al., 2012). Thus, differences in c-Myc levels and the biological context might account for these distinct cellular responses. Furthermore, while RNF4 targets several nuclear proteins, c-Myc is an important target of RNF4 in MDA-MB-231 cells. Yet, the ability of RNF4 to potentiate c-Myc activity may involve other aspects beyond an impact on Myc stability. For example, RNF4 enhances Myc transcriptional activity to a greater extent than an experimental increase in c-Myc protein to a similar level, and the deleterious effects of RNF4 knockdown in MDA-MB-231 cells can partially, but not fully, be rescued by c-Myc overexpression.

## RNF4 Forms a Feed-Forward Loop that Enhances Wnt and Notch Signaling

In response to Wnt ligands,  $\beta$ -catenin directly induces the expression of *c-Myc*. *c-Myc* then induces the expression of the *RNF4* gene, which is a direct *c-Myc* target, and is also induced by Wnt/ $\beta$ -catenin (Reymann and Borlak, 2008; Dutta-Simmons et al., 2009). Once induced, RNF4 enhances Wnt signaling in two molecular ways. First, it inactivates the Groucho/TLE co-repressor alleviating repression of Wnt targets. Second, it stabilizes the key co-activator of the pathway  $\beta$ -catenin and its target *c-Myc* protein. Thus, both mechanisms: RNF4-mediated stabilization of  $\beta$ -catenin and *c-Myc*, and the suppression of Groucho/TLE activity enhance and prolong Wnt signaling (model in Figure 6I).

Similarly, RNF4 establishes a feed-forward loop potentiating Notch signaling. An important and direct target of Notch/NICD is again *c-Myc* (Weng et al., 2006). Subsequently, RNF4 stabilizes NICD and *c-Myc*, and enhances Notch-dependent transcriptional activity (model in Figure 6J).

These feed-forward loops established by RNF4 are important in development. For example, the phenotype observed in the *Drosophila* wing of a Notch gain-of-function mutant is suppressed by reducing the dose of *Dgrn* (Barry et al., 2011). Likewise, this mechanism may also be important to maintain the malignant phenotypes of intestinal tumors and T cell leukemia where *c-Myc* collaborates with Wnt and Notch signaling, respectively (Sansom et al., 2007; Weng et al., 2006).

### A Vital Role for RNF4 in Cancer

RNF4 is essential for the proliferation and survival of aggressive cancer cells and its expression potentiates tumorigenesis in culture. Supporting these observations, we were not able to generate viable null RNF4 homozygous colon cancer cells by CRISPER/CAS9 genome editing. RNF4 is not frequently mutated in breast and colon cancer (<http://cancer.sanger.ac.uk/cosmic>), and *RNF4* mRNA level is elevated in only 3% of colon cancer specimens of the Sanger collection. However, high levels of *RNF4* mRNA correlate with poor outcome of ER<sup>+</sup> luminal “type A” breast cancer patients. Moreover, RNF4 protein levels are elevated in 30% of colon carcinoma samples, but not in normal human intestinal tissues or benign adenomas. The upregulation of *RNF4* mRNA and protein in cancer is in agreement with RNF4’s ability to stabilize and potentiate the transcriptional activity of oncogenes like *c-Myc*,  $\beta$ -catenin, and *c-Jun*. Thus, in defined epithelial cancers, RNF4 fits into a class of genes collectively termed ‘non-oncogene addiction’ genes (NOA; Luo et al., 2009). These act often in a tissue- and cancer-specific manner. They encode proteins that alone are not tumorigenic, but are essential to maintain the tumorigenic phenotypes. “NOA” genes are less significant for the viability of normal cells, making them attractive for cancer therapy. Future studies will determine whether RNF4-positive tumors define a distinct subset of tumors.

## EXPERIMENTAL PROCEDURES

### Materials

Full list of antibodies, plasmids, and primers used in this study can be found in the Supplemental Information.

### Methods

**Cell Lines**—Human HEK293T, MDA-1833, MDA-MB231, HCT116, SW620, and SW480 cells were obtained from the American Type Culture Collection. MDA-MB231<sup>shSAE2Dox</sup> cells were a kind gift of Thomas F. Westbrook (Baylor College of Medicine).

**shRNA Design, Production, and Targeting**—Generation of constitutive pLKO-based shRNF4 (C1, C2) scrambled (scshRNA; control) Dox-induced expression of pInducer-RNF4 system (control, RNF4, RNF4<sup>C159A</sup>, RNF4<sup>ARM</sup>), and conditional miR-30-based RNF4 knockdown (shRNF4-1<sup>Dox</sup>, ShRNF4-2<sup>Dox</sup>, and Renilla control) are described in the Supplemental Experimental Procedures.

**qPCR Analysis**—qPCR analysis was performed as described in Heuberger et al. (2014).

**Flow Cytometry, Cell-Cycle Analysis, MTT Proliferation, Cancer Sphere Assay, and Colony Formation in Soft Agar**—Cell-cycle and apoptosis analyses was performed using the FACS-Calibur (BD Biosciences) system and FlowJo software (Tree Star). MTT assays were performed using MTT solution (Sigma-Aldrich) and processed according to the manufacturer's instructions and Stat Fax 2100 ELISA reader. Colony and sphere formation were performed similar to that described in Staller et al. (2001) and Fang et al. (2016).

**Protein Stability Assays**—Protein stability in steady state and dynamic CHX chase and <sup>35</sup>S-Met pulse/chase experiments were performed as previously described (Abed et al., 2011; Trausch-Azar et al., 2015). Pulse labeling was 10 min for c-Myc and 30 min for NICD at 37°C. Immunoprecipitation of c-Myc and FLAG-NICD proteins was performed using α-c-Myc (N262, 1:50) and α-Flag (1:200), respectively.

Kinase inhibitors were added for 4 hr. Conditional shRNA expression was induced using 200 ng/ml doxycycline for 36 hr before cell harvesting. Cells were lysed in RIPA buffer supplemented with protease and phosphatase inhibitors, and lysates were resolved over SDS-PAGE. Proteins were identified using indicated antibodies and chemiluminescence (Image-Quant LAS4000).

**TNFα-Induced IκBα Degradation**— $3 \times 10^5$  cells/ml HEK293T cells were seeded in 6-well plates and transfected the next day with indicated vectors. 24 hr post-transfection, 40 ng/ml TNFα was added to induce IκBα degradation. Protein levels were determined by western blot analysis.

**Wnt and Myc Luciferase Reporter Assays and Notch Co-culture Reporter Assays**—Reporter assays were performed as previously described in Abed et al. (2011).

Where indicated, LiCl was added for 12 hr at a final concentration of 15 mM to activate the TOP-reporter. TNF $\alpha$  was added for 6 hr at 2.5 ng/ml to activate the NF $\kappa$ B reporter. For testing RNF4 on Notch-dependent activation, co-culture assays were performed as in Ong et al. (2008).

**In Vitro GST-Binding Assays**—Binding assays were performed as previously described (Abed et al., 2011) by using bacterial expressed purified and immobilized GST, GST-RNF4, GSTRNF4<sup>ARM</sup>, GST-Dgrn, and GST-RNF4<sup>SIM</sup> proteins, as well as the indicated in-vitro-translated proteins. For substrate de-phosphorylation, the indicated proteins were pre-incubated with CIP or buffer for 20 min.

**Ubiquitylation and SUMOylation in Cells**—Ubiquitylation and SUMOylation were performed as previously described (Abed et al., 2011; Trausch-Azar et al., 2015). Where indicated, cells were incubated with 40  $\mu$ M MG132 for 4 hr and subjected to hot lysis, followed by immune precipitation. Where His-Ub was used, cells were lysed in 1 ml guanidine-hydrochloride buffer, followed by binding to Ni-NTA beads (QIAGEN) in the presence of 20 mM imidazole. Ubiquitylated and SUMOylated proteins were visualized via SDS-PAGE gels followed by western blotting.

**Pathology**—Biopsies were from the Institute of Pathology, RAMBAM Healthcare Haifa. Paraffin-embedded sections were prepared and immunostained with indicated antibodies using BenchMark XT system. A certified GI pathologist confirmed diagnosis of each case (under the Helsinki committee approval number 0239-12-RMB).

**Survival Analyses of Breast Cancer Patients**—Analyses of systemically-untreated cohort of breast cancer patients including Kaplan-Mayer survival curves were performed using [kmpplot.com](http://kmpplot.com) (<http://kmpplot.com/analysis/index.php?p=service&start=1>). n = 324, p < 0.068 HR = 1.66 (1.15–2.4), and the following criteria: ER+, Type: “luminal, A” systemically untreated patients.

**Digital Image Processing**—Original ECL-were captured using LAS4000. Original TIFF files were transferred to Photoshop<sup>TM</sup> software. If required, using “levels” option at the Photoshop<sup>TM</sup> menu the entire file was adjusted to point to subtle differences. Subsequently, the relevant part of the image was copied to CanvasX<sup>TM</sup> graphic program to generate the illustration. At this stage only proportional adjustments in scale were made. No other processing were performed. Files were saved as high resolution CanvasX<sup>TM</sup> TIFF file. Where indicated signal quantification was performed using Image-Quant<sup>TM</sup>.

**Statistical Analysis**—Statistical analysis, SEM and t test comparisons were performed using Graph-Pad Prism and ANOVA software. In all experiments, significance is as follows: \*\*\*\* p < 0.0001, \*\*\* p < 0.001, \*\* p < 0.05, \* p < 0.01.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## REFERENCES

- Abed M, Barry KC, Kenyagin D, Koltun D, Phippen TP, Delrow JJ, Parkhurst SM, Orian A. Degringolade, a SUMO-targeted ubiquitin ligase, inhibits Hairy/Groucho-mediated repression. *EMBO J.* 2011; 30:1289–1301. [PubMed: 21343912]
- Amit S, Hatzubai A, Birman Y, Andersen JS, Ben-Shushan E, Mann M, Ben-Neriah Y, Alkalay I. Axin-mediated CKI phosphorylation of beta-catenin at Ser 45: a molecular switch for the Wnt pathway. *Genes Dev.* 2002; 16:1066–1076. [PubMed: 12000790]
- Barry KC, Abed M, Kenyagin D, Werwie TR, Boico O, Orian A, Parkhurst SM. The *Drosophila* STUbL protein Degringolade limits HES functions during embryogenesis. *Development.* 2011; 138:1759–1769. [PubMed: 21486924]
- Bassermann F, Eichner R, Pagano M. The ubiquitin proteasome system - implications for cell cycle control and the targeted treatment of cancer. *Biochim. Biophys. Acta.* 2014; 1843:150–162. [PubMed: 23466868]
- Choi HK, Choi KC, Yoo JY, Song M, Ko SJ, Kim CH, Ahn JH, Chun KH, Yook JI, Yoon HG. Reversible SUMOylation of TBL1-TBLR1 regulates  $\beta$ -catenin-mediated Wnt signaling. *Mol. Cell.* 2011; 43:203–216. [PubMed: 21777810]
- Ciechanover A, DiGiuseppe JA, Bercovich B, Orian A, Richter JD, Schwartz AL, Brodeur GM. Degradation of nuclear oncoproteins by the ubiquitin system *in vitro*. *Proc. Natl. Acad. Sci. USA.* 1991; 88:139–143. [PubMed: 1846034]
- Cinnamon E, Paroush Z. Context-dependent regulation of Groucho/TLE-mediated repression. *Curr. Opin. Genet. Dev.* 2008; 18:435–440. [PubMed: 18721877]
- Clevers H, Loh KM, Nusse R. Stem cell signaling. An integral program for tissue renewal and regeneration: Wnt signaling and stem cell control. *Science.* 2014; 346:1248012. [PubMed: 25278615]
- Dutta-Simmons J, Zhang Y, Gorgun G, Gatt M, Mani M, Hideshima T, Takada K, Carlson NE, Carrasco DE, Tai YT, et al. Aurora kinase A is a target of Wnt/beta-catenin involved in multiple myeloma disease progression. *Blood.* 2009; 114:2699–2708. [PubMed: 19652203]
- Fang L, Zhu Q, Neuenschwander M, Specker E, Wulf-Goldenberg A, Weis WI, von Kries JP, Birchmeier W. A small-molecule antagonist of the  $\beta$ -catenin/TCF4 interaction blocks the self-renewal of cancer stem cells and suppresses tumorigenesis. *Cancer Res.* 2016; 76:891–901. [PubMed: 26645562]
- Farrell AS, Sears RC. MYC degradation. *Cold Spring Harb Perspect Med.* 2014 [March 1, 2014] Published online. <http://dx.doi.org/10.1101/cshperspect.a014365>.
- Flotho A, Melchior F. SUMOylation: a regulatory protein modification in health and disease. *Annu Rev Biochem.* 2013; 82:357–385. [PubMed: 23746258]
- Fryrear KA, Guo X, Kerscher O, Semmes OJ. The Sumo-targeted ubiquitin ligase RNF4 regulates the localization and function of the HTLV-1 oncoprotein Tax. *Blood.* 2012; 119:1173–1181. [PubMed: 22106342]
- González-Prieto R, Cuijpers SA, Kumar R, Hendriks IA, Vertegaal AC. c-Myc is targeted to the proteasome for degradation in a SUMOylation-dependent manner, regulated by PIAS1, SENP7 and RNF4. *Cell Cycle.* 2015; 14:1859–1872. [PubMed: 25895136]
- Grocock LM, Nie M, Prudden J, Moiani D, Wang T, Cheltsov A, Rambo RP, Arvai AS, Hitomi C, Tainer JA, et al. RNF4 interacts with both SUMO and nucleosomes to promote the DNA damage response. *EMBO Rep.* 2014; 15:601–608. [PubMed: 24714598]

- Guzzo CM, Berndsen CE, Zhu J, Gupta V, Datta A, Greenberg RA, Wolberger C, Matunis MJ. RNF4-dependent hybrid SUMO-ubiquitin chains are signals for RAP80 and thereby mediate the recruitment of BRCA1 to sites of DNA damage. *Sci. Signal.* 2012; 5:ra88. [PubMed: 23211528]
- Häkli M, Lorick KL, Weissman AM, Jänne OA, Palvimo JJ. Transcriptional coregulator SNURF (RNF4) possesses ubiquitin E3 ligase activity. *FEBS Lett.* 2004; 560:56–62. [PubMed: 14987998]
- Hay-Koren A, Caspi M, Zilberberg A, Rosin-Arbesfeld R. The EDD E3 ubiquitin ligase ubiquitinates and up-regulates beta-catenin. *Mol. Biol. Cell.* 2011; 22:399–411. [PubMed: 21118991]
- Heuberger J, Kosel F, Qi J, Grossmann KS, Rajewsky K, Birchmeier W. Shp2/MAPK signaling controls goblet/paneth cell fate decisions in the intestine. *Proc. Natl. Acad. Sci. USA.* 2014; 111:3472–3477. [PubMed: 24550486]
- Holland JD, Klaus A, Garratt AN, Birchmeier W. Wnt signaling in stem and cancer stem cells. *Curr. Opin. Cell Biol.* 2013; 25:254–264. [PubMed: 23347562]
- Hu XV, Rodrigues TM, Tao H, Baker RK, Miraglia L, Orth AP, Lyons GE, Schultz PG, Wu X. Identification of RING finger protein 4 (RNF4) as a modulator of DNA demethylation through a functional genomics screen. *Proc. Natl. Acad. Sci. USA.* 2010; 107:15087–15092. [PubMed: 20696907]
- Hunter T. Protein kinases and phosphatases: the yin and yang of protein phosphorylation and signaling. *Cell.* 1995; 80:225–236. [PubMed: 7834742]
- Iwai K. Diverse roles of the ubiquitin system in NF- $\kappa$ B activation. *Biochim. Biophys. Acta.* 2014; 1843:129–136. [PubMed: 23523932]
- Kessler JD, Kahle KT, Sun T, Meerbrey KL, Schlabach MR, Schmitt EM, Skinner SO, Xu Q, Li MZ, Hartman ZC, et al. A SUMOylation-dependent transcriptional subprogram is required for Myc-driven tumorigenesis. *Science.* 2012; 335:348–353. [PubMed: 22157079]
- Koo BK, Spit M, Jordens I, Low TY, Stange DE, van de Wetering M, van Es JH, Mohammed S, Heck AJ, Maurice MM, Clevers H. Tumour suppressor RNF43 is a stem-cell E3 ligase that induces endocytosis of Wnt receptors. *Nature.* 2012; 488:665–669. [PubMed: 22895187]
- Kopan R. Notch signaling. *Cold Spring Harb. Perspect. Biol.* 2012; 4:a011213. [PubMed: 23028119]
- Kuo CY, Li X, Kong XQ, Luo C, Chang CC, Chung Y, Shih HM, Li KK, Ann DK. An arginine-rich motif of ring finger protein 4 (RNF4) oversees the recruitment and degradation of the phosphorylated and SUMOylated Kriippel-associated box domain-associated protein 1 (KAP1)/TRIM28 protein during genotoxic stress. *J. Biol. Chem.* 2014; 289:20757–20772. [PubMed: 24907272]
- Levanon D, Goldstein RE, Bernstein Y, Tang H, Goldenberg D, Stifani S, Paroush Z, Groner Y. Transcriptional repression by AML1 and LEF-1 is mediated by the TLE/Groucho corepressors. *Proc. Natl. Acad. Sci. USA.* 1998; 95:11590–11595. [PubMed: 9751710]
- López-Otín C, Hunter T. The regulatory crosstalk between kinases and proteases in cancer. *Nat. Rev. Cancer.* 2010; 10:278–292. [PubMed: 20300104]
- Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. *Cell.* 2009; 136:823–837. [PubMed: 19269363]
- Maher MT, Mo R, Flozak AS, Peled ON, Gottardi CJ. Beta-catenin phosphorylated at serine 45 is spatially uncoupled from beta-catenin phosphorylated in the GSK3 domain: implications for signaling. *PLoS ONE.* 2010; 5:e10184. [PubMed: 20419129]
- Morin PJ, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of  $\beta$ -catenin-Tcf signaling in colon cancer by mutations in  $\beta$ -catenin or APC. *Science.* 1997; 275:1787–1790. [PubMed: 9065402]
- Ong CT, Sedy JR, Murphy KM, Kopan R. Notch and presenilin regulate cellular expansion and cytokine secretion but cannot instruct Th1/Th2 fate acquisition. *PLoS ONE.* 2008; 3:e2823. [PubMed: 18665263]
- Popovic D, Vucic D, Dikic I. Ubiquitination in disease pathogenesis and treatment. *Nat. Med.* 2014; 20:1242–1253. [PubMed: 25375928]
- Poukka H, Aarnisalo P, Santti H, Jänne OA, Palvimo JJ. Coregulator small nuclear RING finger protein (SNURF) enhances Sp1- and steroid receptor-mediated transcription by different mechanisms. *J. Biol. Chem.* 2000; 275:571–579. [PubMed: 10617653]



- Reymann S, Borlak J. Transcription profiling of lung adenocarcinomas of c-myc-transgenic mice: identification of the c-myc regulatory gene network. *BMC Syst. Biol.* 2008; 2:46. [PubMed: 18498649]
- Sansom OJ, Meniel VS, Muncan V, Phesse TJ, Wilkins JA, Reed KR, Vass JK, Athineos D, Clevers H, Clarke AR. Myc deletion rescues *Apc* deficiency in the small intestine. *Nature.* 2007; 446:676–679. [PubMed: 17377531]
- Sears R, Nuckolls F, Haura E, Taya Y, Tamai K, Nevins JR. Multiple Ras-dependent phosphorylation pathways regulate Myc protein stability. *Genes Dev.* 2000; 14:2501–2514. [PubMed: 11018017]
- Sriramachandran AM, Dohmen RJ. SUMO-targeted ubiquitin ligases. *Biochim. Biophys. Acta.* 2014; 1843:75–85. [PubMed: 24018209]
- Staller P, Peukert K, Kiermaier A, Seoane J, Lukas J, Karsunky H, Möröy T, Bartek J, Massagué J, Hänel F, Eilers M. Repression of p15INK4b expression by Myc through association with Miz-1. *Nat. Cell Biol.* 2001; 3:392–399. [PubMed: 11283613]
- Swatek KN, Komander D. Ubiquitin modifications. *Cell Res.* 2016; 26:399–422. [PubMed: 27012465]
- Trausch-Azar JS, Abed M, Orian A, Schwartz AL. Isoform-specific SCF<sup>(Fbw7)</sup> ubiquitination mediates differential regulation of PGC-1 $\alpha$ . *J. Cell. Physiol.* 2015; 230:842–852. [PubMed: 25204433]
- van de Wetering M, Oosterwegel M, Dooijes D, Clevers H. Identification and cloning of TCF-1, a T lymphocyte-specific transcription factor containing a sequence-specific HMG box. *EMBO J.* 1991; 10:123–132. [PubMed: 1989880]
- Welcker M, Orian A, Jin J, Harper WJ, Eisenman RN, Clurman BE. The Fbw7 tumor suppressor regulates glycogen synthase kinase 3 phosphorylation-dependent c-Myc protein degradation. *Proc. Natl. Acad. Sci. USA.* 2004; 101:9085–9090. [PubMed: 15150404]
- Weng AP, Millholland JM, Yashiro-Ohtani Y, Arcangeli ML, Lau A, Wai C, Del Bianco C, Rodriguez CG, Sai H, Tobias J, et al. c-Myc is an important direct target of Notch1 in T-cell acute lymphoblastic leukemia/lymphoma. *Genes Dev.* 2006; 20:2096–2109. [PubMed: 16847353]
- Wickliffe KE, Williamson A, Meyer HJ, Kelly A, Rape M. K11-linked ubiquitin chains as novel regulators of cell division. *Trends Cell Biol.* 2011; 21:656–663. [PubMed: 21978762]
- Winston JT, Strack P, Beer-Romero P, Chu CY, Elledge SJ, Harper JW. The SCFbeta-TRCP-ubiquitin ligase complex associates specifically with phosphorylated destruction motifs in IkappaBalpha and beta-catenin and stimulates IkappaBalpha ubiquitination in vitro. *Genes Dev.* 1999; 13:270–283. [PubMed: 9990852]
- Wu B, Piloto S, Zeng W, Hoverter NP, Schilling TF, Waterman ML. Ring Finger Protein 14 is a new regulator of TCF/beta-catenin-mediated transcription and colon cancer cell survival. *EMBO Rep.* 2013; 14:347–355. [PubMed: 23449499]
- Xie Y, Rubenstein EM, Matt T, Hochstrasser M. SUMO-independent *in vivo* activity of a SUMO-targeted ubiquitin ligase toward a short-lived transcription factor. *Genes Dev.* 2010; 24:893–903. [PubMed: 20388728]
- Yaron A, Hatzubai A, Davis M, Lavon I, Amit S, Manning AM, Andersen JS, Mann M, Mercurio F, Ben-Neriah Y. Identification of the receptor component of the IkappaBalpha-ubiquitin ligase. *Nature.* 1998; 396:590–594. [PubMed: 9859996]
- Yuan WC, Lee YR, Lin SY, Chang LY, Tan YP, Hung CC, Kuo JC, Liu CH, Lin MY, Xu M, et al. K33-linked polyubiquitination of coronin 7 by Cul3-KLHL20 ubiquitin E3 ligase regulates protein trafficking. *Mol. Cell.* 2014; 54:586–600. [PubMed: 24768539]
- Ziv I, Matiuhin Y, Kirkpatrick DS, Erpapazoglou Z, Leon S, Pantazopoulou M, Kim W, Gygi SP, Haguenaer-Tsapis R, Reis N, et al. A perturbed ubiquitin landscape distinguishes between ubiquitin in trafficking and in proteolysis. *Mol Cell Proteomics.* 2011; 10:M111. 009753.

**In Brief**

Ubiquitylation targets many oncogenes for degradation. Thomas et al. show that ubiquitylation by RNF4 stabilizes and enhances the activity of key oncogenes. RNF4 translates transient phosphorylation signal(s) into long-term protein stabilization, potentiating oncogenic signaling and the tumor phenotype. High RNF4 levels in epithelial cancers are correlated with poor survival.

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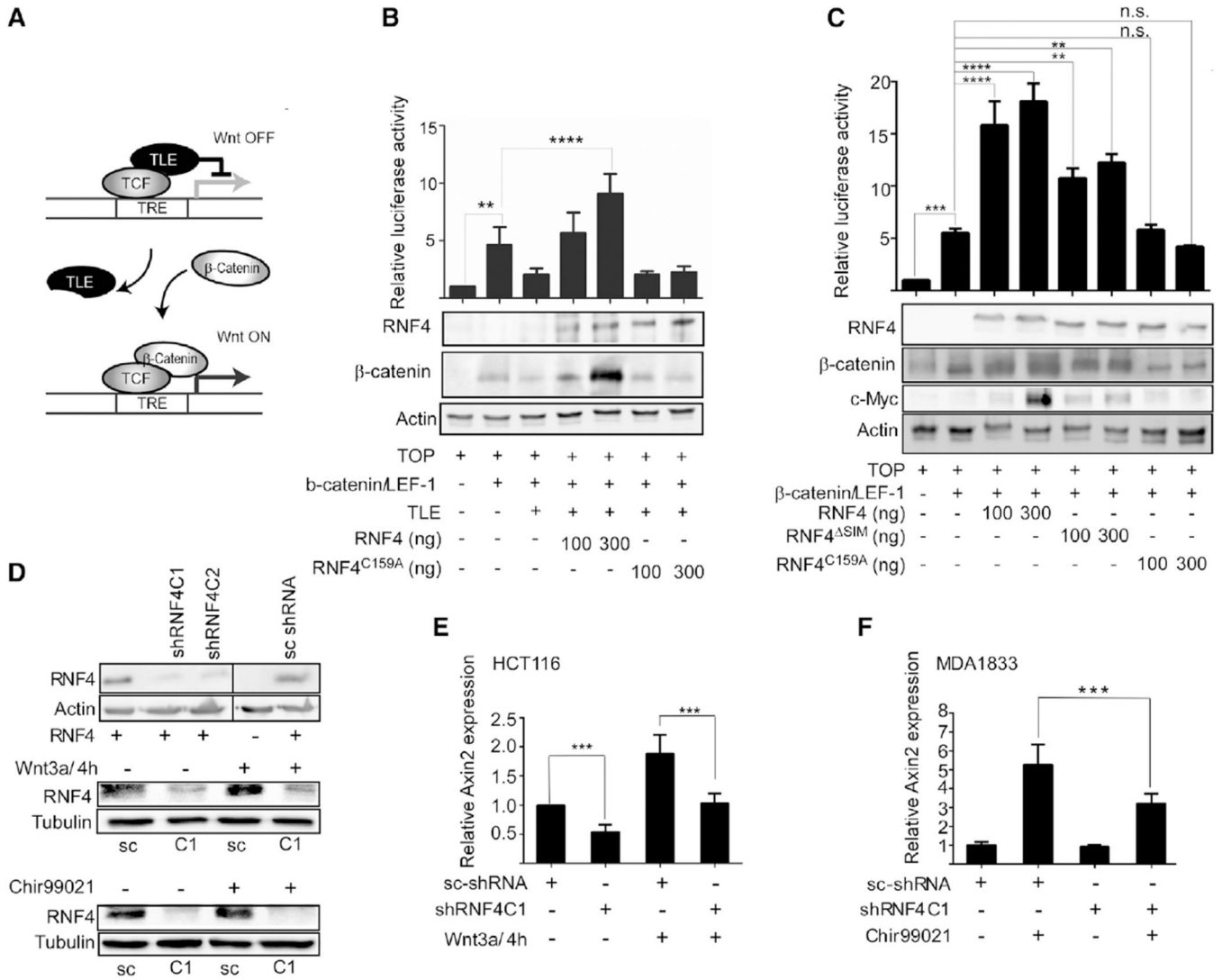
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**Highlights**

- RNF4 stabilizes and enhances the activity of short-lived oncogenes
- Stabilization requires substrate phosphorylation and atypical ubiquitylation
- RNF4 is essential for cancer cell survival
- High RNF4 levels are correlated with reduced survival in epithelial tumors



**Figure 1. RNF4 Enhances Transcriptional Activity of the Wnt/β-Catenin Pathway**  
 (A) Schematic diagram of Wnt target gene activation by displacement of the co-repressor TLE by β-catenin. TRE, TCF-responsive element. Data shown are mean ± SE from three independent experiments performed in triplicate.  
 (B and C) Wnt/β-catenin-luciferase reporter assays in HEK293T cells transfected with the indicated plasmids, and western blot analyses of indicated proteins in extract used in these assays. RNF4, but not the RNF4 RING mutant (hRNF4<sup>C159A</sup>), alleviates TLE1-mediated repression of β-catenin-induced activation of the TOPFLASH reporter (B). Enhancement of transcription by RNF4 requires its RING domain, but not its SIM, motifs (C).  
 (D) Western blot analysis of RNF4 protein levels. Upper: HEK293T cells were transfected with HA-RNF4 cDNA and infected with two independent constitutive RNF4 shRNAs (C1, C2) or a scrambled control (sc-shRNA). Vertical black lines indicate two non-relevant lanes omitted. Middle: endogenous RNF4 protein level in HCT116 colon cancer cells infected with either sc and shRNF4 (C1) coding lentiviral vectors used in (E). Lower: similar analyses of endogenous RNF4 protein in MDA-1833 breast cancer cells used in (F).

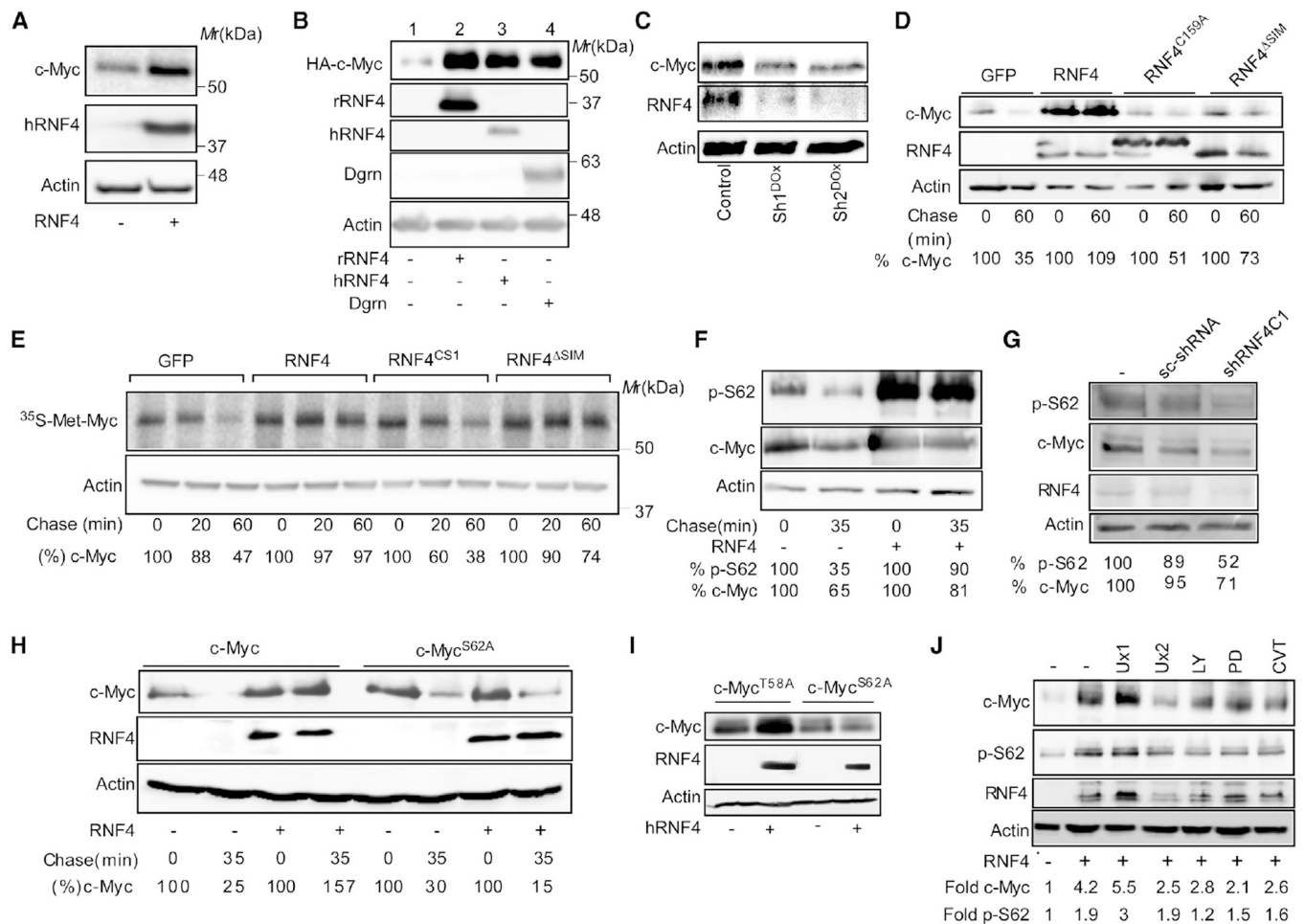
(E and F) qPCR analysis of endogenous *Axin2* mRNA transcripts levels following activation of Wnt signaling in the presence or absence of RNF4 shRNA. Data shown are mean  $\pm$  SE from three independent experiments. *Axin2* mRNA transcripts in HCT116 colon cancer cells infected with the indicated shRNAs with or without Wnt3a treatment for four hours (E). *Axin2* mRNA transcripts of MDA-1833 breast cancer cells without or with a 4-hr incubation with the GSK-inhibitor Chir-99021 (2.5  $\mu$ M) (F).

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**Figure 2. RNF4 Stabilizes p-Ser62 c-Myc**

(A) Endogenous c-Myc levels in HEK293T cells expressing plasmids coding for GFP (“-”) or human RNF4.

(B) Steady-state levels of exogenous c-Myc protein in cells overexpressing GFP (lane 1) or rat, human, or *Drosophila melanogaster* RNF4 orthologs (lanes 2–4, respectively).

(C) Endogenous c-Myc and RNF4 protein levels are reduced following expression of doxycycline-induced (200 ng/ml) shRNF4 (Sh1<sup>Dox</sup>, Sh2<sup>Dox</sup>), but not control shRNA (Renilla). Actin serves as loading control.

(D) Cycloheximide (CHX)-chase for the indicated times, followed by western blot of HEK293T cells expressing HA-c-Myc, and the indicated RNF4 coding plasmids.

(E) <sup>35</sup>S-Met pulse-chase analysis of exogenous c-Myc in HEK293T cells. Expression of rRNF4, but not of the RING mutant rRNF4<sup>CS1</sup>, stabilizes c-Myc protein levels, while rRNF4<sup>SIM</sup> expression partially stabilizes c-Myc levels.

(F and G) Phosphorylation of Ser62-c-Myc is required for its stabilization by RNF4. CHX-chase for the indicated times, followed by western blot analysis using pSer62 specific antibody (upper, CST #13748), or the 9E10  $\alpha$ -c-Myc antibody (middle) (F). Steady-state levels of endogenous proteins in extracts derived from MD-MB 231 cells expressing shRNF4C1 or control (sc-shRNA) using  $\alpha$ -pSer62-c-Myc, pan  $\alpha$ -c-Myc (9E10), and  $\alpha$ -RNF4 antibodies (G).



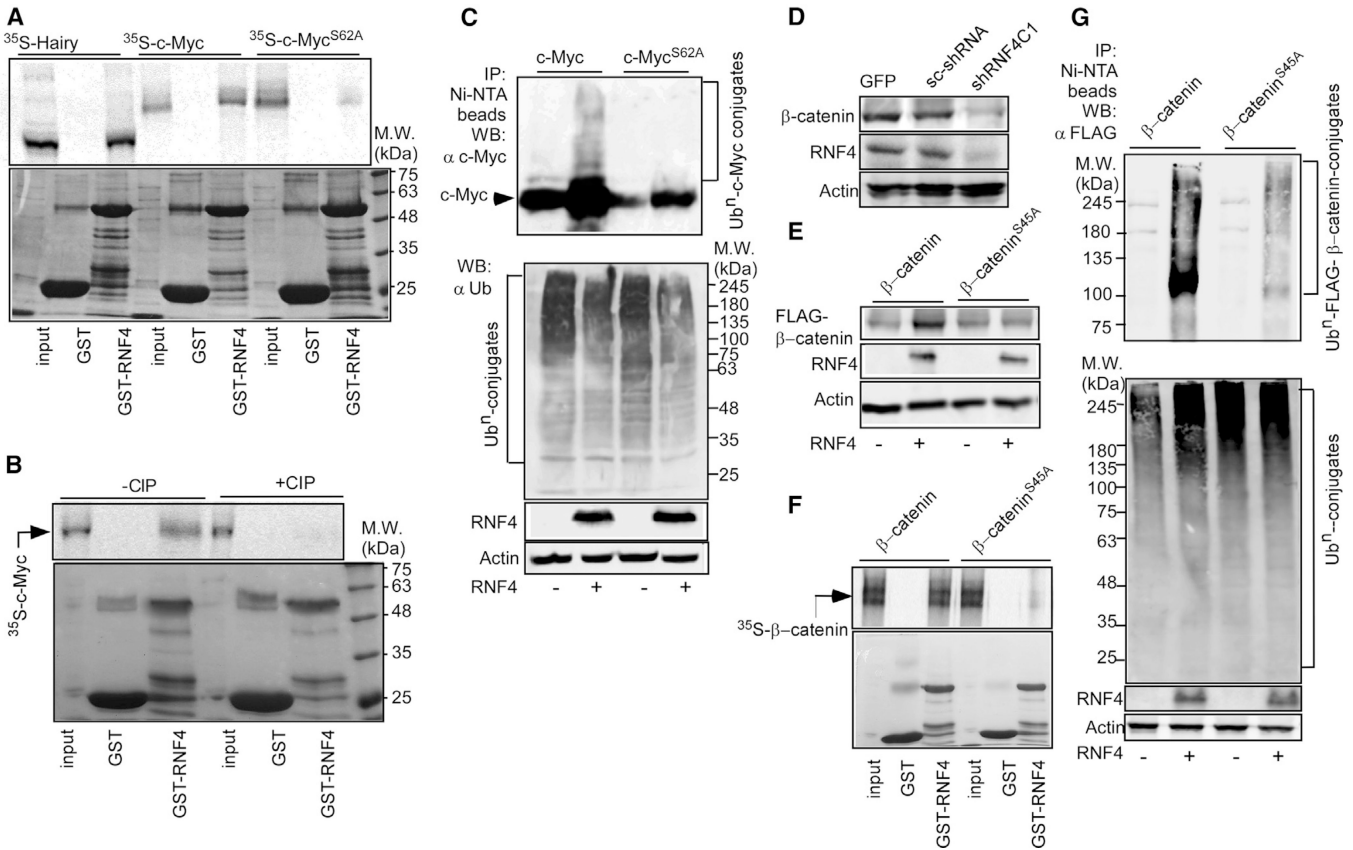
(H and I) Expression of the indicated plasmids in HEK293T cells, followed by western analysis. c-Myc<sup>S62A</sup> is not stabilized by RNF4 in dynamic CHX-chase assay (H). c-Myc<sup>T58A</sup>, but not Myc<sup>S62A</sup>, protein levels are stabilized by RNF4 at steady state (I). (J) Chemical inhibition of kinases involved in Ser62-c-Myc phosphorylation impairs RNF4-dependent c-Myc stabilization. The level of indicated proteins was determined using western blot analysis. Fold: relative change in c-Myc and p-Myc levels. Ux1, 7.5  $\mu$ M U0126; Ux2, 15  $\mu$ M U0126; PD, 10  $\mu$ M PD98059, are MEK inhibitors. LY, 10  $\mu$ M LY2228820 is a MAP38 inhibitor; CVT, 10  $\mu$ M CVT313 inhibits Cdk2.

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**Figure 3. RNF4-Mediated Stabilization Requires Binding and Ubiquitylation of Phosphorylated pSer62-c-Myc and pSer45-β-Catenin**

(A) In-vitro-translated (IVT) labeled  $^{35}\text{S}$ -Met-Hairy (positive control) and  $^{35}\text{S}$ -Met-c-Myc bind to GST-RNF4, while  $^{35}\text{S}$ -Met-c-Myc<sup>S62A</sup> binds weakly to GST-RNF4.

(B) Mock-treated, but not CIP-treated,  $^{35}\text{S}$ -Met-c-Myc binds to GST-RNF4 (CIP, calf intestinal phosphatase).

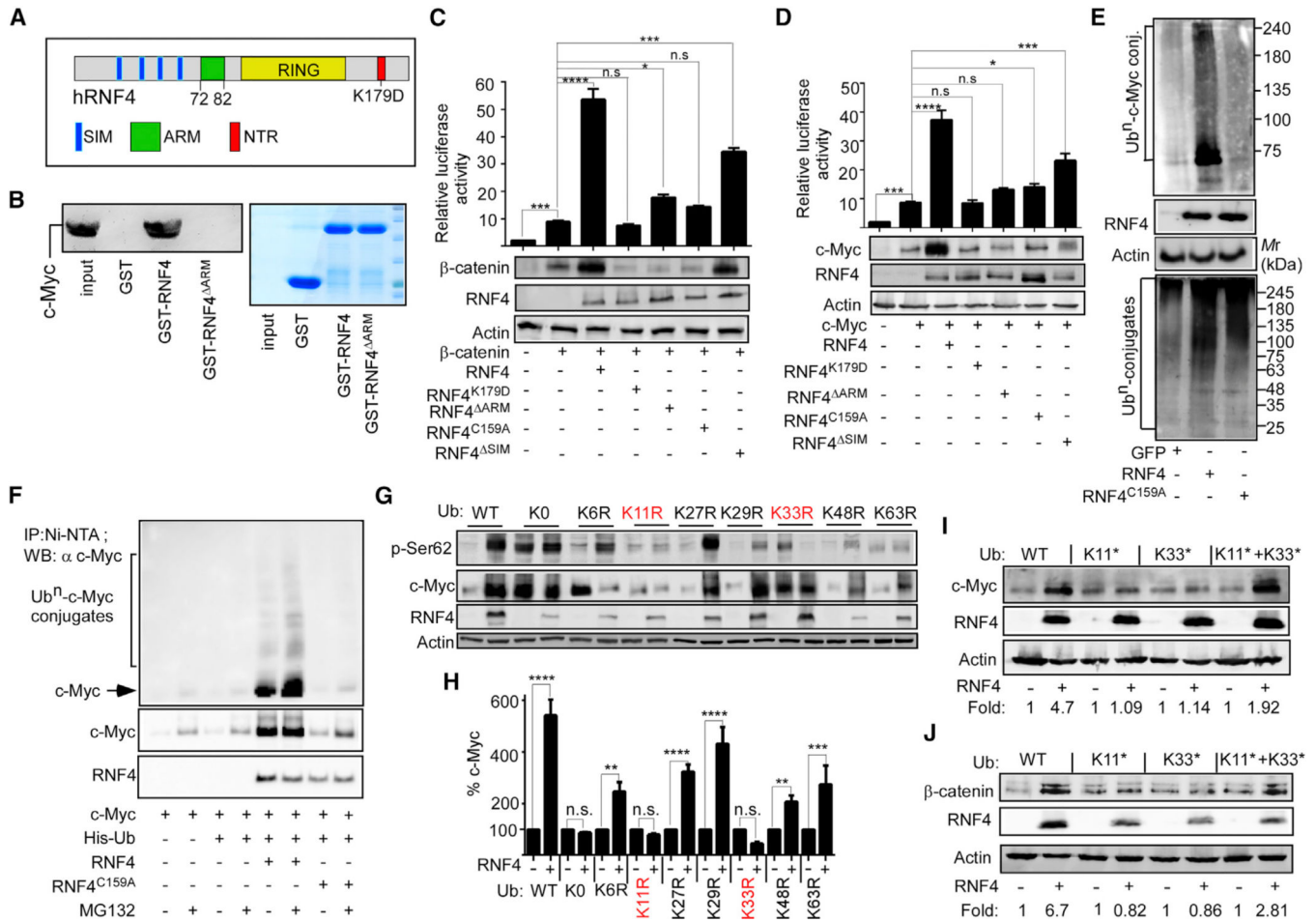
(C) Upper: RNF4 ubiquitylates c-Myc and to a lesser extent c-Myc<sup>S62A</sup> in HEK293T cells. Cells were transfected with indicated plasmids, and His-Ub~proteins were recovered on Ni-NTA agarose, followed by western blot analysis with  $\alpha$  c-Myc antibody. Lower: overall ubiquitylation determined using a  $\alpha$ -Ub antibody. Actin serves as loading control.

(D) Steady-state levels of endogenous  $\beta$ -catenin and RNF4 in extracts derived from MD-MB231 cells expressing shRNF4(C1) or control sc-shRNA.

(E) FLAG- $\beta$ -catenin, but not FLAG- $\beta$ -catenin<sup>S45A</sup>, is stabilized by RNF4 in HEK293T cells.

(F) IVT  $^{35}\text{S}$ -Met- $\beta$ -catenin, but not  $^{35}\text{S}$ -Met  $\beta$ -catenin<sup>S45A</sup>, binds to GST-RNF4. In (A), (B), and (F) 5% input is shown, and lower gels are Coomassie staining of the gel.

(G) Upper: RNF4 ubiquitylates  $\beta$ -catenin, but not  $\beta$ -catenin<sup>S45A</sup>. Ubiquitylated FLAG- $\beta$ -catenin was detected using  $\alpha$ -FLAG antibody. Lower: overall ubiquitylation was determined by using a  $\alpha$ -Ub antibody.



**Figure 4. RNF4-Dependent Stabilization Requires the ARM Domain and K179 within RNF4 as well as the Catalysis of K11/ K33-Containing Polyubiquitin Chains**

(A) Schematic diagram of hRNF4 (not to scale): SIM, SUMO-interacting motif; ARM, arginine-rich motif; RING, really interesting gene; NTR, nucleosome-targeting region. (B) Left: in-vitro-translated (IVT) c-Myc binds to GST-RNF4, but not to GST-RNF4<sup>ΔARM</sup>. Right: protein levels of the GST-fusion proteins used as determined by Coomassie blue staining. (C and D) β-catenin (C) and c-Myc (D) luciferase reporter assays in HEK293T cells transfected with the indicated plasmids. Data shown are mean ± SE from three independent experiments performed in triplicates. Lower: western blot analyses of steady-state levels of β-catenin, c-Myc, and RNF4 in representative extracts. Actin serves as loading control. (E) Endogenous c-Myc is ubiquitylated by RNF4, but not by the RING mutant RNF4<sup>C159A</sup>. Upper: HEK293T cells were transfected with the indicated plasmids. Endogenous c-Myc was immunoprecipitated using α-c-Myc antibody (#sc789), and ubiquitylated c-Myc was detected using α-Ub antibody. Lower: overall ubiquitylation determined using a α-Ub antibody. (F) Expression of RNF4, but not of RNF4<sup>C159A</sup>, results in c-Myc ubiquitylation. The proteasome inhibitor MG132 was added as indicated, c-Myc~Ub conjugates were recovered using Ni-NTA resin and detected using α-c-Myc antibody (9E10).

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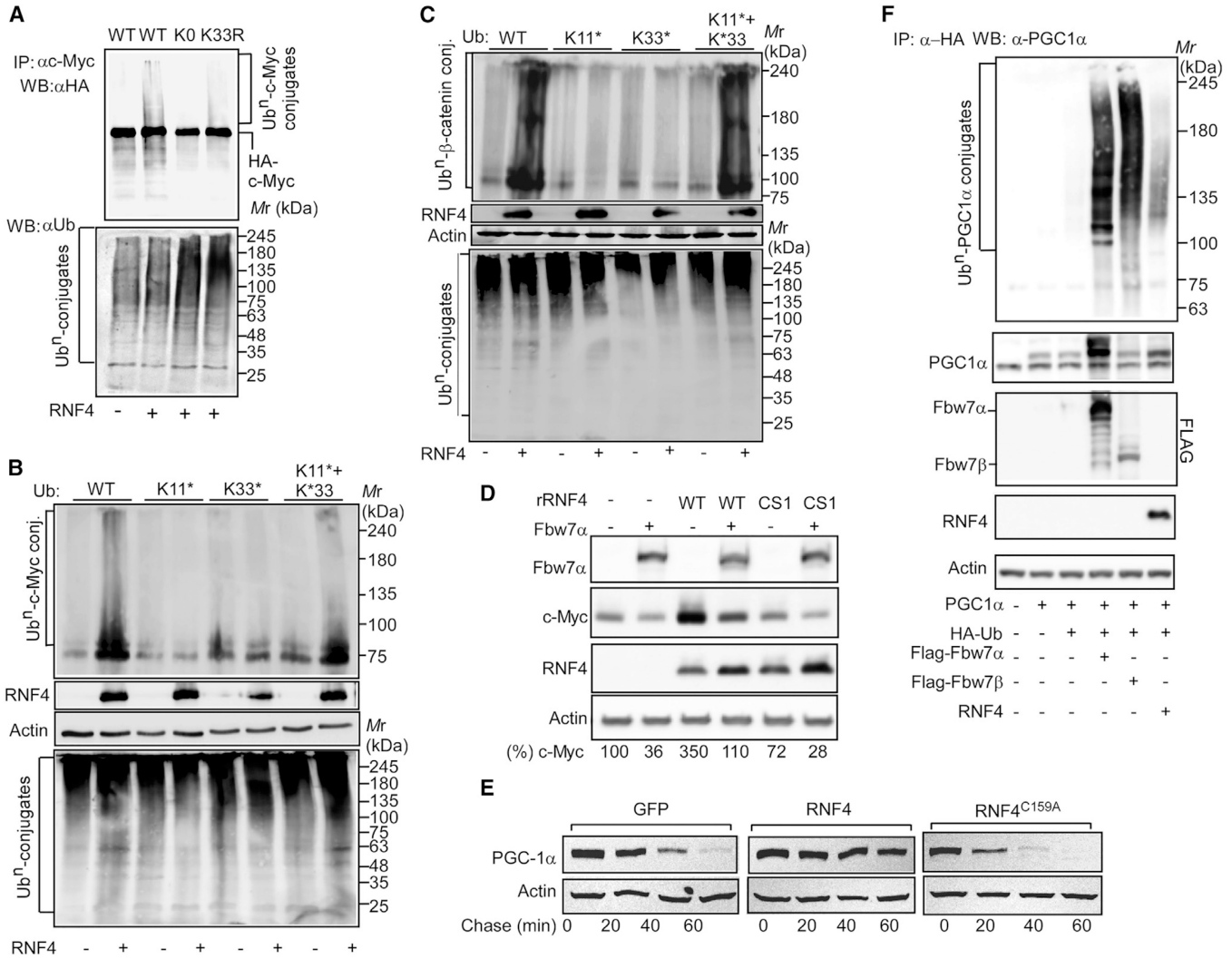
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(G and H) c-Myc stabilization requires RNF4-dependent catalysis of K11/K33 containing polyubiquitin chains. Western blot analysis of the indicated proteins in extracts derived from HEK293T cells transfected with c-Myc and GFP or RNF4 expression vectors. Where indicated, cells were transfected with HA-tagged wild-type ubiquitin or mutants where a specific Lys was replaced by Arg (K#R). K0 is an ubiquitin molecule in which all internal Lys residues were replaced with Arg. c-Myc protein level was determined using indicated antibodies (G). Quantitation of the effect of ubiquitin mutants on RNF4-dependent c-Myc stabilization using 9E10 antibody (Data shown are mean  $\pm$  SE, n=6) (H).

(I and J) Steady-state protein levels of c-Myc (I),  $\beta$ -catenin (J), and RNF4 in cells transfected with the indicated ubiquitin mutants. UbK11\* and UbK33\* are ubiquitin molecules that contain a single lysine where all other lysine residues are replaced with Arg. Fold: relative change in c-Myc and  $\beta$ -catenin protein levels.



**Figure 5. K11 and K33 within Ubiquitin Are Required for RNF4-Dependent Ubiquitylation, and RNF4 Does Not Inhibit c-Myc Degradation by Fbw7α**

(A–F) Western blot analyses of extracts derived from HEK293T cells transfected with the indicated vectors.

(A) Upper: c-Myc polyubiquitylation by RNF4 requires polyubiquitylation and K33 Lys residue within ubiquitin. Lower panel: Overall ubiquitylation determined using a α-Ub antibody.

(B and C) Co-expression of K11\* and K33\* within ubiquitin is sufficient for RNF4-dependent ubiquitylation. Upper: Cells were transfected with plasmids coding for c-myc (B) or β-catenin (C), and where indicated with RNF4 as well as wild-type ubiquitin or ubiquitin mutants that harbor single Lys residues (UbK11\* and UbK33\*). Lower: Overall ubiquitylation determined using a α-Ub antibody.

(D) Expression of SCF<sup>Fbw7</sup> reduces c-Myc protein levels in control and RNF4-expressing cells.

(E and F) RNF4 stabilizes and ubiquitylates PGC1α. (E) CHX-chase experiment (0–60 min) in cells expressing plasmids coding for PGC1α, GFP, RNF4, or RNF4<sup>C159A</sup>, followed by

western blot analysis of PGC1 $\alpha$  and actin (loading control). (F) PGC1 $\alpha$  is ubiquitylated by RNF4. Cells were transfected with HA-ubiquitin and the indicated plasmids. Ubiquitylation was detected similar to that described in (A).

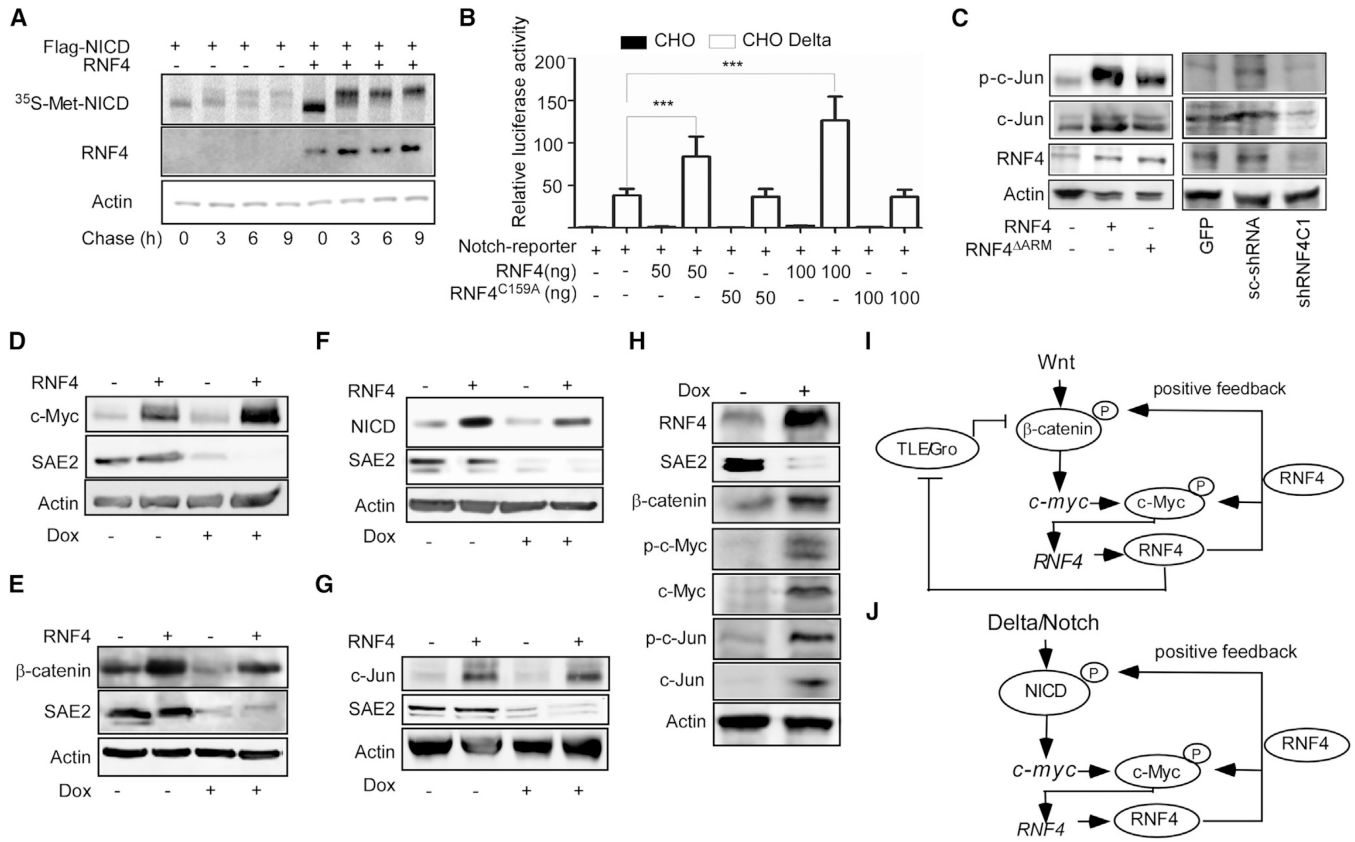
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**Figure 6. RNF4 Stabilizes Short-Lived Oncoproteins and Enhances Notch-Dependent Transcriptional Activation Independent of De Novo SUMOylation**

(A) <sup>35</sup>S-Met pulse chase analysis of FLAG-NICD in MK4 cells expressing indicated plasmids, followed by α-Flag-IP.

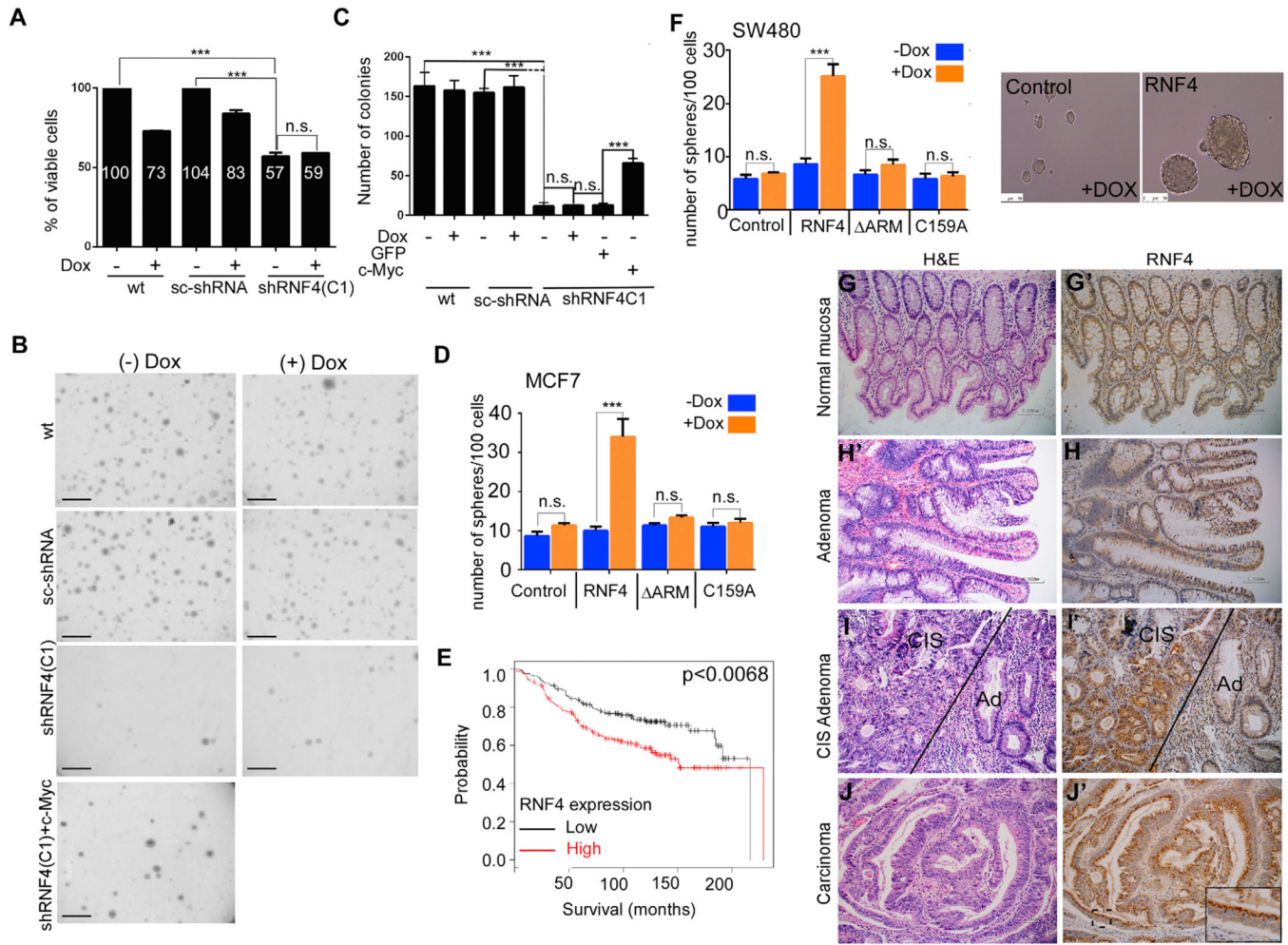
(B) RNF4, but not RNF4<sup>C159A</sup>, enhances Delta-dependent Notch-induced activation of the TP1-Luciferase canonical Notch reporter in a co-culture assay. Data shown are mean ± SE from three independent experiments. MK4 cells were co-cultured either with CHO-IRES-GFP (control, black bars) or CHO-Delta-IRES-GFP (white bars), as indicated.

(C) Left: RNF4, but not RNF4<sup>ARM</sup>, stabilizes p-c-Jun. Right: c-Jun and p-c-Jun protein levels in MDA-MB231 cells expressing shRNF4(C1) or sc-shRNA.

(D–G) Protein levels of HA-c-Myc (D), FLAG-β-catenin (E), FLAG-NICD (F), His-c-Jun (G), and endogenous SAE2 in MDA-MB231<sup>shSAE2Dox</sup> cells. Cells were transfected with indicated plasmids in the presence or absence of 200 ng/ml doxycycline (Dox). Actin serves as loading control.

(H) Western blot analyses of endogenous proteins in MDA-MB231<sup>shSAE2Dox</sup> co-expressing lenti-viral Dox-induced RNF4.

(I and J) Proposed model for RNF4-dependent enhancement of Wnt (I) and Notch (J) signaling. Genes are depicted in *italics*, and proteins are embedded in ovals. Ⓟ indicates phosphorylation.



**Figure 7. RNF4 Is Essential for Survival and Potentiates Tumorous Phenotype of Cancer Cells and Its mRNA and Protein Levels Are Elevated in Subtypes of Breast and Colon Adenocarcinoma**

(A) MTT proliferation assay in MDA-MB231<sup>shSAE2Dox</sup> cells infected with indicated shRNAs, where Dox-induced the expression of SAE2 shRNA (Dox+). Data shown are mean ± SE from three independent experiments.

(B and C) RNF4, but not SAE2, is required for colony formation in soft agar of MDA-MB231<sup>shSAE2Dox</sup> cells. (B) Representative colony formation assays in soft agar. Dox (Dox+) activates shSAE2, inactivating SAE2. Co-expression of c-Myc along with RNF4 inactivation partially restores the ability of the cells to form colonies. Scale bar represents 3.5 mm. (C) Quantification of colony formation assays. Data shown are mean ± SE from three independent experiments (n = 3).

(D) Sphere formation in non-adherent conditions of MCF7 breast cancer cells is enhanced by expression of Dox-induced RNF4, but not in control, RNF4<sup>ARM</sup>, or RNF4<sup>C159A</sup>. Data shown are mean ± SE from three independent experiments.

(E) Kaplan-Meier survival curves of ER-positive “type A” luminal breast cancer patients with low (black) or high RNF4 (red) mRNA levels (p = 0.0068, n = 324).

(F) Left: sphere formation in non-adherent conditions of SW480 colon cancer cells is enhanced by expression of Dox-induced RNF4, but not in control, RNF4<sup>ARM</sup>, or RNF4<sup>C159A</sup>. Data shown are mean  $\pm$  SE from three independent experiments performed in triplicates. Right: microscopic image of representative spheres. Scale bar represents 50  $\mu$ m. (G–J') RNF4 protein levels in patient-derived colon biopsies. (G–J) H&E staining. (G'–J') Immunostaining with  $\alpha$ -RNF4. (G–G') Normal colonic mucosa. (H–H') Colonic adenoma. (I and I') High-grade colonic adenoma with in situ carcinoma (CIS). Note the elevated RNF4 levels left to the dotted line, where the cancerous part of the lesion. (J–J') Adenocarcinoma of the colon. The dotted black square is shown in the inset. (J'). Scale bar represents 10  $\mu$ m.