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RNF4-dependent oncogene activation by protein stabilization

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RNF4-Dependent Oncogene Activation by Protein Stabilization

Graphical Abstract

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

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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.
RNF4-Dependent Oncogene Activation by Protein Stabilization

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INTRODUCTION

 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 oncoproteic transcription factors, including β-catenin, Myc, c-Jun, and the Notch intracellular-domain (N-ICD) protein. RNF4 enhances the transcriptional activity of these factors, 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.

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 oncoproteic transcription factors, including β-catenin, Myc, c-Jun, and the Notch intracellular-domain (N-ICD) protein. RNF4 enhances the transcriptional activity of these factors, 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.
translocates to the nucleus. Subsequently, nuclear β-catenin displaces the co-repressor Gro/TLE from TCF4, which results in activation of TCF4/β-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/β-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 β-catenin protein. In addition, RNF4 stabilizes other phosphorylated nuclear oncoproteins, including c-Myc, NICD, c-Jun, and PGC1α. 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/β-Catenin-Dependent Transcriptional Activity

In Drosophila, the RNF4 ortholog Degringolade (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 β-catenin-induced activation of the TOP reporter. Co-expression of RNF4, but not the mutant RNF4C159A which lacks ubiquitylation activity, alleviated this repression and enhanced transcriptional activation (Figure 1B). RNF4 enhanced both TCF/β-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 β-cateninY33A, a constitutively active oncogenic form of β-catenin (Figure S1B; Morin et al., 1997). This potentiation of transcriptional activation by RNF4 depends on the binding of the TCF/β-catenin activation complex to DNA, as RNF4 had no impact on a reporter that lacks TCF binding sites (TOPFLASH; TOP), 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/β-catenin target genes in various cancer cell lines. Constitutive RNF4 small hairpin RNAs (shRNAs) (shRNF4-C1, shRNF4-C2), but not control (sc-shRNA), 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/β-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 β-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 β-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 β-catenin and its target c-Myc. We found that RNF4 expression increased the levels of β-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 β-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 35S-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/Mpkl 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 35S-Met-labeled c-Myc in vitro. This binding was greatly reduced when we used the non-phosphorylatable 35S-Met-c-Myc562A 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-Myc562A mutant was poorly ubiquitylated (Figure 3C, upper). Moreover, we found that RNF4 stabilizes Ser45 phosphorylated β-catenin. Similar to the case of Ser62-c-Myc, phosphorylation of Ser45 within β-catenin enhances its activity (Maher et al., 2010), but also primes β-catenin for GSK-dependent phosphorylation and degradation (Amit et al., 2002). We found that RNF4 is required for maintaining endogenous β-catenin protein level, and that phosphorylation of β-catenin Ser45 is required for stabilization, binding, and ubiquitylation of β-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-β-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 β-Catenin Stabilization

To understand how RNF4 interacts directly with its substrates, we defined the structural motifs within RNF4 that recognize phosphorylated c-Myc and β-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 β-catenin, the ARM deletion mutant, RNF4ΔARM, 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; RNF4K179D), which mediates its interaction with nucleosomes, failed to stabilize c-Myc or β-catenin (Figures 4A, 4C, and 4D; Groocock et al., 2014). In addition, both the ARM domain and K179 were required for RNF4-dependent enhancement of β-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, RNF4ΔSIM, which cannot bind to SUMOylated proteins, enhanced β-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 indicated plasmids, and His-Ub–proteins were recovered on Ni-NTA agarose, followed by western blot analysis with α c-Myc antibody. Lower: overall ubiquitylation determined using a α-Ub antibody. Actin serves as loading control.

Figure 3. RNF4-Mediated Stabilization Requires Binding and Ubiquitylation of Phosphorylated pSer62-c-Myc and pSer45-β-Catenin
(C) Upper: RNF4 ubiquitylates c-Myc and to a lesser extent c-MycS62A 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 α c-Myc antibody. Lower: overall ubiquitylation determined using a α-Ub antibody. Actin serves as loading control.
(D) Steady-state levels of endogenous β-catenin and RNF4 in extracts derived from MD-MB231 cells expressing shRNF4(C1) or control sc-shRNA.
(E) FLAG-β-catenin, but not FLAG-β-cateninS45A, is stabilized by RNF4 in HEK293T cells.
(F) IVT 35S-Met-β-catenin, but not 35S-Met-β-cateninS45A 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 β-catenin, but not β-cateninS45A. Ubiquitylated FLAG-β-catenin was detected using α-FLAG antibody. Lower: overall ubiquitylation was determined by using a α-Ub antibody.

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and SCF$^{bTRCP}$, respectively (Welcker et al., 2004; Winston et al., 1999). We asked whether RNF4 compromises the activity of these ligases. Transfection of SCF$^{Fbw7}$ in HEK293T cells decreased c-Myc protein levels 3-fold (Figure 5D). Co-expression of RNF4 and SCF$^{Fbw7}$ also reduced c-Myc levels 3-fold compared to expression of RNF4 alone. Thus, RNF4 does not interfere with SCF$^{Fbw7}$-dependent c-Myc degradation. Similarly, an established assay for SCF$^{bTRCP}$ 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, and had no effect on TNF$\alpha$-induced NF-$\kappa$B transcription activation (Figures S3A–S3C). We also found that RNF4 does

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$^{\DeltaARM}$. 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$^{C159A}$. 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$^{C159A}$, 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).

(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). Quantification of the effect of ubiquitin mutants on RNF4-dependent c-Myc stabilization using 9E10 antibody (Data shown are mean ± SE, n=6) (H).

(I and J) Steady-state protein levels of c-Myc (I), β-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 β-catenin protein levels.
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\textsuperscript{Fbw7}. These include, PGC1\textalpha, (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\textsuperscript{C159A}, enhanced Delta-dependent Notch-induced activation of the TP1-Luciferase canonical Notch 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\textsuperscript{a} repressor, was described (Xie et al., 2010). Therefore, we determined whether RNF4-dependent stabilization requires 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\textsuperscript{DoxSAE2shRNA} 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, β-catenin, 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 β-catenin (Figure 6E), the ability of RNF4 to stabilize β-catenin, and enhance β-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\textsuperscript{DoxSAE2shRNA} cells with lentiviral RNF4 and quantified the levels of endogenous proteins by Western blotting (Figure 6H).

Figure 6. RNF4 Stabilizes Short-Lived Oncoproteins and Enhances Notch-Dependent Transcriptional Activation Independent of De Novo SUMOylation
(A) \textsuperscript{35}S-Met pulse chase analysis of FLAG-NICD in MK4 cells expressing indicated plasmids, followed by α-Flag-IP.
(B) RNF4, but not RNF4\textsuperscript{C159A}, 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\textsuperscript{DARM}, 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\textsuperscript{SAE2shRNA} 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\textsuperscript{DoxSAE2shRNA} 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 italic, and proteins are embedded in ovals. © indicates phosphorylation.
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 RNF4ΔARM or RNF4C159A enhanced sphere formation of MCF7 breast cancer cells (Figure 6H). We further verified that the essential role of RNF4 in these cells is mediated, in part, by its effect on c-Myc. Another example in which RNF4 recognizes a phosphorylated substrate is the binding of RNF4 to the C-terminal region of the Wnt co-receptor LRP6. RNF4 directly associates with phosphorylated LRP6 and potentiates the expression of Wnt target genes, such as c-Myc. This interaction is mediated by the ARM domain of RNF4 and is independent of SUMOylation. Additionally, RNF4 binds to the C-terminal domain of the β-catenin and enhances its transcriptional activity, which is critical for the proliferation and survival of cancer cells.

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 underlying 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 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 RNF4ΔARM or RNF4C159A 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 7J). 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 β-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.
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 β-catenin by SCFTRCP. The ubiquitin ligases RNF43, and RNF14 modulate Wnt pathway activity but do not alter β-catenin stability or bind to β-catenin directly (Koo et al., 2012; Wu et al., 2013). A third ligase, EDD, stabilizes β-catenin but requires phosphorylation of Ser33 of β-catenin; the onco-
genic β-cateninS33A mutant is unaffected by EDD (Hay-Koren et al., 2011). However, RNF4 enhanced β-cateninS33A activity, indicating that EDD and RNF4 target different pools of β-catenin modified by distinct mechanisms. Moreover, the observations...
that RNF4 enhances the activity of cancerous-related β-catenin mutants may offer a strategy to target cancers where the degradation of β-catenin is compromised.

Similar to ubiquitylation, SUMOylation regulates the Wnt pathway. We show here that targeting the SUMO E1 subunit SAE2 reduced β-catenin protein level and impaired β-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 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, β-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/β-catenin (Reyumann and Borlak, 2008; Dutta-Simmons et al., 2009). Once induced, RNF4 enhances Wnt signaling in two molecular ways. First, it inactivates the Groucho/TLE corepressor alleviating repression of Wnt targets. Second, it stabilizes the key co-activator of the pathway β-catenin and its target c-Myc protein. Thus, both mechanisms: RNF4-mediated stabilization of β-catenin and c-Myc, and the suppression of Groucho/TLE activity enhance and prolong Wnt signaling (model in Figure 6J).

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/CASS9 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+ 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, β-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 and H1299 (DOX) cells were a kind gift of Thomas F. Westbrook (Baylor College of Medicine).

**shRNA Design, Production, and Targeting**

Generation of constitutive pLKO-based shRNA4 (C1, C2) scrambled (sc-shRNA; control) Dox-induced expression of pInducer-RNF4 system (control, RNF4, RNF4Δ1–319A, RNF4Δ1–457A, and conditional miR-30-based RNF4 knock-down (shRNF4-1Δ53, shRNF4-2Δ53, 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 Stailer et al. (2001) and Fang et al. (2016).

**Protein Stability Assays**

Protein stability in steady state and dynamic CHX chase and 35S-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 × 10^6 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, LCI was added for 12 hr at a final concentration of 15 mM to activate the TOP-reporter. TFNα was added for 6 hr at 2.5 ng/ml to activate the NFκB 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, GST-RNF4ASRM, GST-Dgrn, and GST-RNF4ASHRM 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 μM 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 kmplot.com (http://kmplot.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™ software. If required, using “levels” option at the Photoshop™ menu the entire file was adjusted to point to subtle differences. Subsequently, the relevant part of the image was copied to CanvasX™ graphic program to generate the illustration. At this stage only proportional adjustments in scale were made. No other processing was performed. Files were saved as high resolution CanvasX™ TIFF file. Where indicated signal quantification was performed using Image-Quant™.

**Statistical Analysis**

Statistical analysis, SEM and t test comparisons were performed using GraphPad Prism and ANOVA software. In all experiments, significance is as follows: *** p < 0.0001, ** p < 0.001, * p < 0.05, † p < 0.1.

**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 potentiates 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 preformed 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., A.B.L., and A.O. wrote the manuscript.

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**REFERENCES**


