NOA- and Degradation-resistant tumors

Amir Orian, *MD/PhD, Stan and Ruth Flinkman Laboratory for Genetic Networks in Development and Cancer*

The nature of degradation-resistant tumors. During cancer development, cancer cells exhibit dramatic stabilization of key regulatory proteins (oncoproteins) that are essential for tumor development and cancer progression. Physiologically in non-transformed cells, the half-life of these proteins is very short (minutes) (1). Yet, in cancer cells, the half-life of oncoproteins is prolonged and they remain in their active state, driving tumorigenesis. Two major reasons are behind this phenomenon:

1. In many cases, mutations of amino acids within short stretches that mediate their recognition to the degradation machinery ("degrons") enable these oncoproteins to evade recognition by the enzymes that mediate their ubiquitin-dependent degradation. Examples of such mutations are Thr58>Ala, which is the common mutation in the c-Myc oncoprotein, and Ser33>Ala in β-catenin (2-4). In both cases, these mutations prevent phosphorylation by the GSK3-beta kinase. These phosphorylations are required for recognition by the SCF^{FbW7} or $SCF^{\beta\text{-}TRCP}$ ubiquitin ligase complexes, respectively, which mediate the ubiquitination and subsequent degradation of the oncoproteins (4, 5).
2. Stabilization as a result of inactivation of enzymes within the degradation machinery that physiologically regulates the degradation of these oncoproteins. For example, FbW7 is the receptor subunit of the SCF^{FbW7} ubiquitin ligase complex. It is an potent tumor suppressor that is inactivated by various mechanisms in many cancers (5). Among the substrates of SCF^{FbW7} are Cyclin E, Notch intracellular domain protein (NICD), c-Jun, PGC-1α and c-Myc. Thus, upon inactivation of FbW7, multiple oncogenes in a single cancer cell that drive G1/S transition are stabilized pathologically resulting in a "degradation-resistant cell." <u>These cells, and subsequently tumors, represent a challenge to target pharmacologically as their tumorigenesis is driven by multiple oncogenic drivers.</u>

Ubiquitin-dependent pathway that stabilizes oncoproteins in cancer. Can the increased stability of oncoproteins be overcome? Our laboratory recently discovered a physiological pathway in cancer cells that confers stability to these nuclear oncoproteins and is essential for cancer cell survival *in vivo* and *in vitro*. A central enzyme within this pathway is the ubiquitin ligase RNF4. While, in many cases, ubiquitination leads to degradation of the ubiquitinated protein, in the case of these oncoproteins, RNF4-dependent ubiquitination stabilizes and increases their half-life and activity (6).

How does RNF4 function? RNF4 belongs to a small family of RING ubiquitin ligases conserved from yeast and flies to humans called SUMO-Targeted Ubiquitin ligases (STUbL), which regulate embryonic development and tissue homeostasis, and are deregulated in cancer (Figure 1; 7, 8). STUbL connect the ubiquitin with the SUMO pathways. At the N-terminal region, STUbL harbor multiple SUMO-interacting motifs that enable binding to poly-SUMOylated proteins, and via the RING domain, they catalyze the ubiquitination of these SUMOylated proteins. For example, arsenic-induced SUMOylation targets the Promyelocytic Leukemia Protein (PML) for degradation. Therefore, in the cases of promyelocytic leukemia, RNF4 function is tumor suppressive (7). However, in epithelial cancers RNF4 activity is oncogenic and its pro-oncogenic function is independent of SUMOylation.

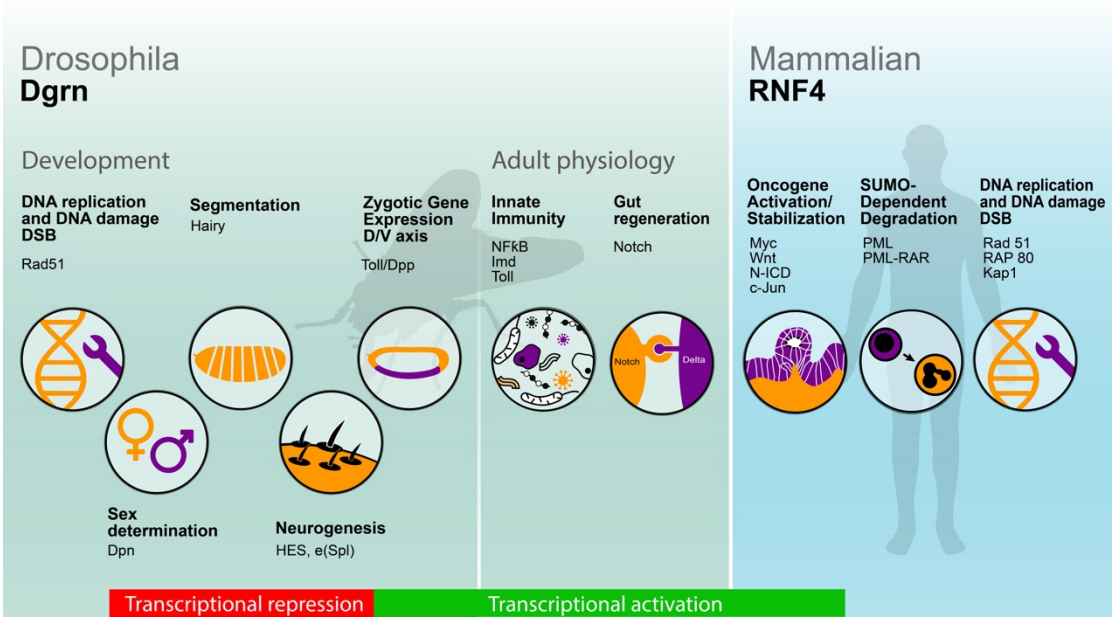

Figure 1: *Processes and key proteins regulated by Dgrn* (fly STUbL) *and RNF4 in Drosophila and humans.* During embryogenesis, Dgrn is required to resolve DSBs, and a similar function was attributed to RNF4 upon DNA damage in mice and humans. In transcription, Dgrn determines co-factors choice during transcriptional repression that is involved in segmentation, sex determination and neurogenesis. Dgrn and RNF4 also enhance transcriptional activation of early zygotic genes, the transcription of AMP genes, as well as Notch-dependent transcription and adult gut regeneration. Likewise, RNF4 stabilizes and potentiates the transcriptional activity of c-Myc, c-Jun and β-catenin promoting tumorigenesis. In contrast, in the context of promyelocytic leukemia, RNF4 ubiquitinates and targets the SUMOylated oncogenic PML-RAR for degradation, which suppresses tumorigenesis (adopted from 8).

Importantly, and in cancer, this RNF4-dependent stabilization pathway is situated and acts upstream to the molecular machinery that ubiquitinates and degrades these proteins. Remarkably, it also stabilizes oncoproteins where the "degrons" are mutated (6). Thus, inhibiting the pathway genetically shortens the oncoproteins' half-life dramatically and is affective on multiple oncogenic substrates within the cancer cell, including the case of degradation-resistant tumors (Figure 2).

Mechanisms of RNF4-dependent protein stabilization. A common denominator of these stabilized oncoproteins is that they are all subjected to phosphorylation by mitogenic kinases. These phosphorylations prolonged the half-life and activity of these oncoproteins in a way that was previously unknown. We found that these phosphorylations, such as Ser62 in c-Myc and Ser 45 in β-catenin, are recognized by RNF4. Mutations abolishing these phosphorylations prevent interaction with the ligase and subsequently stabilization. Interestingly, protein stabilization requires the ability of RNF4 to interact with chromatin/nucleosomes. A point mutation that abolishes RNF4 ability to bind nucleosomes did not support oncoprotein stabilization. Molecular analysis revealed that RNF4-dependent protein stabilization requires RNF4-dependent catalysis of heterotypic mixed ubiquitin chains with internal link of K11 and K33 within the ubiquitin molecule. However, how the formation of mixed ubiquitin chains promotes oncoprotein stabilization is not fully understood and awaits further study. A recent report hinted at a possible mechanism involving interaction with nuclear lamins (9).

Cancer biology and clinical significance of protein stabilization. The nuclear oncoproteins stabilized by RNF4 are transcription factors, and RNF4-dependent stabilization increases their transcriptional activity. In accordance, RNF4 expression promotes the tumorigenic properties of cancer cells in culture and *in vivo*. Moreover, inactivation of RNF4 results in the death of cancer cells and the collapse of tumors *in vivo*, suggesting an addiction to its presence. In patient-derived biopsies, high RNF4 mRNA and protein levels were associated with poorer prognosis. In colon cancer, an elevated RNF4 protein level was observed in 30% of colon adeno carcinoma but not in adenoma. In type A luminal breast cancer, a high level of RNF4 was associated with poorer prognosis (6).

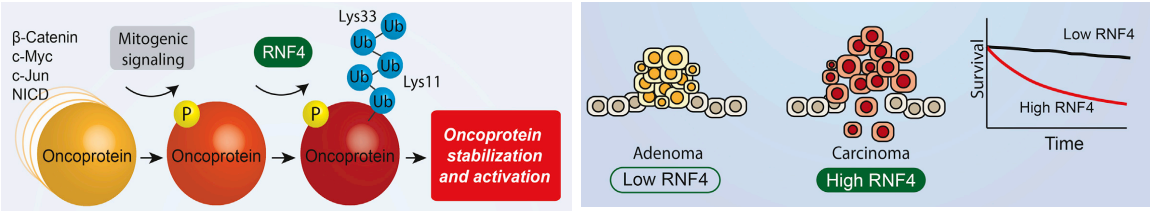

Figure 2: *RNF4 dependent protein stabilization.* RNF4 stabilizes and enhances the activity of short-lived oncogenes. Stabilization requires substrate phosphorylation and atypical ubiquitylation RNF4 is essential for cancer cell survival. High RNF4 levels are correlated with reduced survival in epithelial tumors (adopted from 6).

Targeting degradation-resistant tumors. The biochemical, biological and clinical data suggest that while RNF4 is not oncogenic by itself, it fits well into an emerging class of genes called "non-oncogenic addiction genes" (NOA; 10). These genes are essential in cancer cells but are less important in non-transformed cells. Therefore, NOA genes are attractive molecular targets for future therapeutics. Indeed, an ongoing effort in the laboratory is to fully expose the enzymatic pathway involved in protein stabilization and to develop diagnostic tools for the identification of degradation-resistant tumors and inhibitors to these enzymes.

Related References

1. 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.
2. Wang X, Cunningham M, Zhang X, Tokarz S, Laraway B, Troxell M, Sears RC. Phosphorylation regulates c-Myc's oncogenic activity in the mammary gland. *Cancer Res.* 2011; 71:925-936. PMID 21266350; http//dx.doi.org/10.1158/0008-5472.CAN-10-1032.
3. Farrell AS, Sears RC. MYC degradation. *Cold Spring Harb Perspect Med.* 2014; 4. pii: a014365.
4. Stamos JL, Weis WI. The β-catenin destruction complex. *Cold Spring Harb Perspect Biol.* 2013; 5:a007898. PMID 23569527 http//dx.doi.org/10.1101/cshperspect.a007898.
5. Davis RJ, Welcker M, Clurman BE. Tumor suppression by the Fbw7 ubiquitin ligase: mechanisms and opportunities. *Cancer Cell* 2014; 26:455-464. PMID 25314076; http//dx.doi.org/10.1016/j.ccell.2014.09.01.
6. Thomas JJ, Abed M, Heuberger J, Novak R, Zohar Y, Beltran Lopez AP, Trausch-Azar JS, Ilagan M, Benhamou D, Dittmar G, Kopan R Birchmeier W, Schwartz AL, Orian A. RNF4-dependent oncogene activation by protein stabilization. *Cell Reports* 2016; 16:3388-3400. PMID 27635678 doi.org/10.1016/j.celrep.2016.08.024.
7. Sriramachandran AM, Dohmen RJ. SUMO-targeted ubiquitin ligases. *Biochim Biophys Acta.* 2014; 1843:75-85. PMID 24018209; http//dx.doi.org/10.1016/j.bbamcr.2013.08.022.
8. Abed M, Bitman-Lotan E, Orian A. The biology of SUMO-targeted ubiquitin ligases in Drosophila development, immunity, and cancer. *J. Dev. Biol.* 2018; doi:10.3390/jdb6010002
9. Myant K, Qiao X, Halonen T, Come C, Laine A, Janghorban M, Partanen JI, Cassidy J, Ogg EL, Cammareri P, Laiterä T, Okkeri J, Klefström J, Sears RC, Sansom OJ, Westermarck J. Serine 62-phosphorylated MYC associates with nuclear lamins and its regulation by CIP2A is essential for regenerative proliferation. *Cell Reports* 2015; 12:1019-1031. PMID 26235622; http//dx.doi.org/10.1016/j.celrep.2015.07.003.
10. Nagel R, Semenova EA, Berns A. Drugging the addict: non-oncogene addiction as a target for cancer therapy. *EMBO Rep.* 2016; 5:823-837. PMID 27702988; http//dx.doi.org/ 10.15252/embr.20164303