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Lenalidomide induces degradation of IKZF1 and IKZF3

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Lenalidomide and its analogs, thalidomide and pomalidomide, specifically inhibit growth of mature B-cell lymphomas, including multiple myeloma, and induce interleukin-2 (IL-2) release from T cells. We recently found that this results from activation of the CRBN-CRL4 E3 ubiquitin ligase to degrade the lymphoid transcription factors IKZF1 (Ikaros) and IKZF3 (Aiolos).

Thalidomide was developed in Germany in the 1950s and 60s as a sedative and was broadly used in Europe as an antiemetic in pregnant women. Tragically, the drug caused severe teratogenicity, primarily limb deformations, that affected more than 10,000 children, and it was removed from the market a few years later. In the 1990s, additional properties of thalidomide were described including selective growth inhibition of multiple myeloma cells and modulation of immune cells. Thalidomide stimulates IL-2 production by co-activated T-cells and inhibits tumor necrosis factor (TNF) release from monocytes. Lenalidomide and pomalidomide were developed as more potent so-called immunomodulatory drugs (IMiDs). More than 50 y after the teratogenicity of thalidomide was first described, and 15 y following the first trials of thalidomide in cancer, the molecular basis of the activity of this class of drugs remained unknown until recently.

Using a lenalidomide derivative immobilized to a bead, we found that lenalidomide binds the CRBN-DDB1-CUL4A-ROC1 E3 ubiquitin ligase (CRBN-CRL4).¹ Ito et al. identified the same

complex as the target of thalidomide,² demonstrated that binding of thalidomide inhibited CRBN auto-ubiquitination, and found that the CRBN-CRL4 E3 ligase is responsible for the teratogenicity of thalidomide.²

To identify substrates of the CRBN-CRL4 E3 ligase that mediate the activity of lenalidomide, we applied SILAC (stable isotope labeling of amino acids in cell culture) based quantitative mass spectrometry studies to assess global changes in ubiquitination and protein levels in multiple myeloma cells. Lenalidomide increased the ubiquitination and decreased the protein levels of 2 members of the Ikaros family zinc finger transcription factors, IKZF1 (Ikaros) and IKZF3 (Aiolos), with striking specificity.¹ In addition, we found that both IKZF1 and IKZF3 bind the substrate adaptor, CRBN, and that this interaction is increased in the presence of lenalidomide, suggesting that lenalidomide may cause the CRBN-CRL4 E3 ligase to increase ubiquitination of these transcription factors. Subsequent validation experiments in different cell lines and patient samples demonstrated that lenalidomide, thalidomide, and pomalidomide, all cause

a decrease in the protein levels of both endogenous and ectopically expressed IKZF1 and IKZF3 but not of the other Ikaros family members IKZF2, IKZF4 and IKZF5. *In vitro* ubiquitination reactions of IKZF3 co-immunoprecipitated with CRBN revealed that IKZF1 and IKZF3 are direct substrates of the CRBN-CRL4 E3 ligase.¹ Two other groups independently found that lenalidomide induces ubiquitination of IKZF1 and IKZF3 by using a near genome-wide library of luciferase-fusion proteins to measure protein stability in 293T cells³ and ubiquitination profiling in primary human T cells.⁴

IKZF1 and IKZF3 are central transcriptional regulators of lymphopoiesis.⁵ We found that *IKZF1* and *IKZF3* specific shRNAs or expression of an *IKZF3* dominant negative mutant inhibited the growth and survival of multiple myeloma cell lines. One of the direct transcriptional targets of IKZF1 and IKZF3 in multiple myeloma is *interferon regulatory factor 4* (*IRF4*), a gene that has recently been implicated in the activity of lenalidomide through transcriptional repression.⁶ Overexpression of *IRF4* conferred partial lenalidomide-resistance to multiple myeloma

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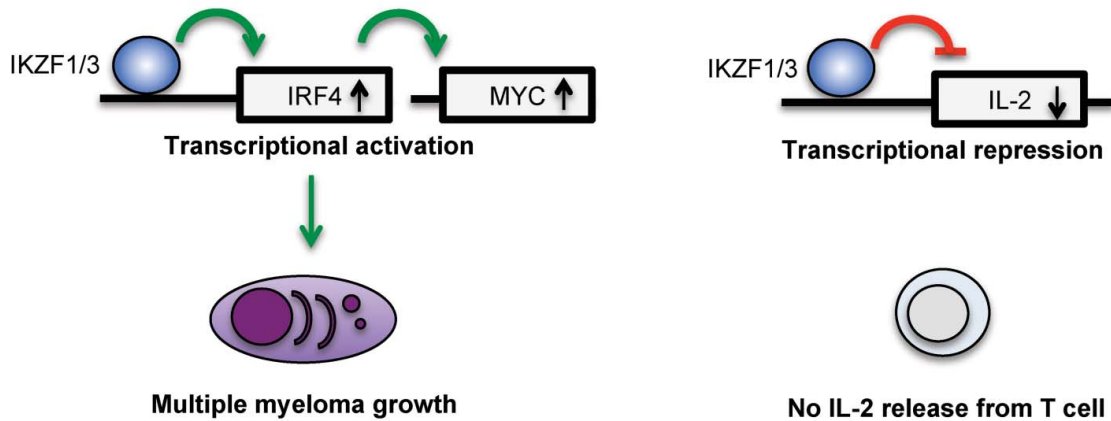
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Normal



Lenalidomide

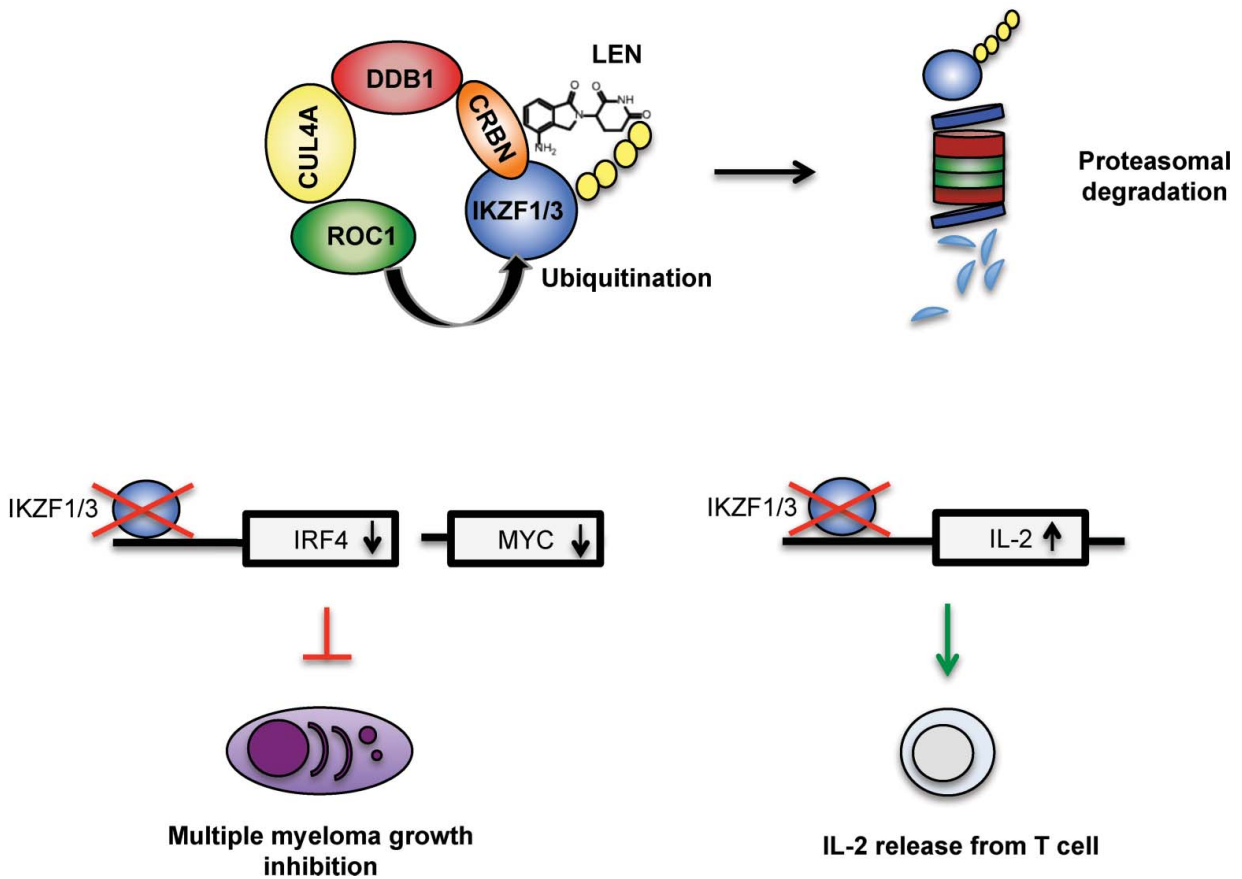


Figure 1. Lenalidomide mode of action. The transcription factors IKZF1 and IKZF3 activate transcription of *IRF4* which is a transcriptional activator of *c-MYC*. IKZF1, IKZF3, *IRF4* and *MYC* form a transcriptional network that is essential for multiple myeloma survival and growth. On the *IL-2* gene locus IKZF1 and IKZF3 are transcriptional repressors. Lenalidomide promotes ubiquitination of IKZF1 and IKZF3 by the CRBN-CRL4 E3 ligase resulting in their proteasomal degradation. As a consequence of IKZF1 and IKZF3 degradation, *IRF4* and *MYC* transcription decrease resulting in growth inhibition of multiple myeloma cells and de-repression of *IL-2* in T cells. Abbreviations: IKZF1 and 3, IKAROS family zinc finger 1 and 3; IL-2, interleukin-2; IRF4, interferon regulatory factor 4.

cell lines, suggesting that it is one of the relevant transcriptional targets of IKZF1 and IKZF3 in this disease. In T-cells, IKZF3 is a transcriptional repressor of IL-2,⁷ and degradation of IKZF3 by lenalidomide leads to an increase of IL-2 expression and release. (Fig. 1)

While IKZF1 and IKZF3 are essential for the survival of multiple myeloma and potentially other mature B-cell malignancies, these proteins are tumor suppressors in acute lymphocytic leukemia (ALL). Deletions, loss-of-function mutations, and dominant-negative mutations of *IKZF1* and *IKZF3* are common somatic genetic lesions in ALL.⁸ Mice with a germline dominant negative *IKZF1* mutation that also inactivates IKZF3 due to heterodimerization fail to generate mature lymphocytes and develop leukemia with a high penetrance.⁵ While *IKZF1* is expressed throughout lymphocytic maturation, including stem cells and early progenitors, *IKZF3* expression is restricted to more differentiated cells. Remarkably,

IKZF3 is essential for the generation of plasma cells, the physiologic counterparts of multiple myeloma.⁹ The effects of IMiDs on lymphocytes are therefore highly consistent with observations made in *IKZF1* and *IKZF3* knockout mice.

In other cell types, IMiDs likely alter the abundance of different proteins through modulation of the activity of the CRBN-CRL4 ubiquitin ligase. Limb deformations, for example, do not occur with germline genetic inactivation of *IKZF1* or *IKZF3*. It is also unlikely that depletion of IKZF1 and IKZF3 can cause the dramatic therapeutic efficacy of lenalidomide in myelodysplastic syndrome (MDS) patients with del(5q). Similarly, IKZF1 and IKZF3 have not been implicated in the cellular pathways that lead to TNF release in monocytes. Identification of the substrates responsible for each of these biological effects could enable the development of more specific drugs that modify ubiquitination of different sets of proteins with notably fewer side effects.

Insights into the molecular basis of lenalidomide activity raise the possibility of a novel class of therapeutic agents that target CRBN-CRL4 or other E3 ubiquitin ligases to modulate degradation of specific proteins. A biological precedent for this approach, in addition to the IMiDs, is the plant hormone auxin. Like the IMiDs, auxin binds to an E3 ubiquitin ligase, SCF-TIR1, and promotes binding and degradation of the transcription factor Aux/IAA.¹⁰ Since both Auxin and IMiDs target transcription factors, a particularly intriguing possibility is that this approach could target other “undruggable” proteins, involved in human diseases. Insights into the structural basis of IMiD binding to CRBN will aid in the rational design of novel small molecule modulators of E3 ubiquitin ligases.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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