In this work, both CAR T-cell infusions employed cellular products derived from the same leukapheresis, suggesting that differential responses cannot be ascribed to intrinsic T-cell defects. Similarly, the protocol used for CAR T-cell manufacturing was the same, suggesting a negligible role for this aspect in explaining the outcomes. Alternatively, differences in the peculiar host environment and disease status that preceded the 2 infusions may impact the outcomes. Clinical trials designed to address the beneficial effect of a second CAR T-cell infusion should explore this crucial aspect.

Assuming that an allogeneic hematopoietic stem cell transplantation is recommended to consolidate long-term responses, this article shows that a second CAR T-cell infusion may help more patients achieve a favorable outcome. In this scenario, additional clinical factors will potentially impact treatment decisions. Tumor CD19 antigen expression profiling after the first treatment failure may impact the decision about whether to proceed with a second infusion of the same CAR T-cell product or opt for an alternative specificity, such as CD22.[6,9]

It has been recently reported that infusing CD22 CAR T cells in patients who achieved remission after CD19 CAR T-cell therapy is effective in consolidating responses,[10] but a formal comparison of a second infusion of CD19 CAR T cells with CD22 CAR T cells will clarify the cost-benefit ratio of this approach.

In summary, despite its retrospective nature and the small size of the patient cohort for each disease entity, this study will contribute to the design of future clinical trials for heavily pre-treated patients who fail first CAR T-cell immunotherapy.

Conflict-of-interest disclosure: The authors declare no competing financial interests. ∎

REFERENCES

1. Gauthier J, Bezerra ED, Hirayama AV, et al. Factors associated with outcomes after a second CD19-targeted CAR T-cell infusion for refractory B-cell malignancies. *Blood*. 2021; 137(3):323-335.

2. Turtle CJ, Hanafi L-A, Berger C, et al. Immunotherapy of non-Hodgkin's lymphoma with a defined ratio of CD8+ and CD4+ CD19-specific chimeric antigen receptor-modified T cells. *Sci Transl Med*. 2016;8(355):355ra116.

3. Gardner RA, Finney O, Annesley C, et al. Intent-to-treat leukemia remission by CD19 CAR T cells of defined formulation and dose in children and young adults. *Blood*. 2017; 129(25):3322-3331.

4. Das RK, Vernau L, Grupp SA, Barrett DM. Naïve T-cell deficits at diagnosis and after chemotherapy impair cell therapy potential in pediatric cancers. *Cancer Discov*. 2019;9(4): 492-499.

5. Fraietta JA, Lacey SF, Orlando EJ, et al. Determinants of response and resistance to CD19 chimeric antigen receptor (CAR) T cell therapy of chronic lymphocytic leukemia. *Nat Med*. 2018;24(5):563-571.

6. Shah NN, Highfill SL, Shalabi H, Yates B. CD4/ CD8 T-cell selection affects chimeric antigen receptor (CAR) T-cell potency and toxicity: updated results from a phase I anti-CD22 CAR T-cell trial. *J Clin Oncol*. 2020;38(17): 1938-1950.

7. Hirayama AV, Gauthier J, Hay KA, et al. The response to lymphodepletion impacts PFS in patients with aggressive non-Hodgkin lymphoma treated with CD19 CAR T cells. *Blood*. 2019;133(17): 1876-1887.

8. Kochenderfer JN, Somerville RPT, Lu T, et al. Lymphoma remissions caused by anti-CD19 chimeric antigen receptor T cells are associated with high serum interleukin-15 levels. *J Clin Oncol*. 2017;35(16): 1803-1813.

9. Fry TJ, Shah NN, Orentas RJ, et al. CD22-targeted CAR T cells induce remission in B-ALL that is naive or resistant to CD19-targeted CAR immunotherapy. *Nat Med*. 2018;24(1):20-28.

10. Pan J, Zuo S, Deng B, et al. Sequential CD19-22 CAR T therapy induces sustained remission in children with r/r B-ALL. *Blood*. 2020;135(5): 387-391.

HEMATOPOIESIS AND STEM CELLS

Comment on Okamoto et al, page 336

Fanconi anemia, put to sleep

**Andrew J. Deans** | St. Vincent's Institute of Medical Research

In this issue of *Blood*, Okamoto et al describe how reduced levels of *Schlafen* protein SLFN11 can rescue the DNA damage sensitivity in a Fanconi anemia (FA) cell line. They propose SLFN11 as a potential target for treatment of FA.[1]

FA is the most common inherited bone marrow failure syndrome and is also associated with an elevated predisposition to cancer. All of the phenotypes of FA are associated with unrepaired DNA interstrand crosslink (ICL) damage. Thus, FA cells are hypersensitive to endogenous byproducts of metabolism such as formaldehyde[2] or chemical agents such as cisplatin that act to covalently crosslink the 2 strands of DNA. ICLs are a potent barrier to DNA replication because the strands cannot be separated for duplication. This leads to stalled replication forks (see figure). In FA cells, something else happens; not only is replication blocked by the ICL, but newly synthesized DNA adjacent to the replication fork becomes actively degraded.[3] For this reason, it is thought that a major cellular function of FA gene products is fork protection.[4]

Although the exact mechanics of fork protection are enigmatic, they center on 2 processes. One process is the monoubiquitination-induced clamping of FANCD2:FANCI at DNA adjacent to the fork.[5] The other process is RAD51-, BRCA1-, and BRCA2-dependent homologous recombination–mediated stabilization of the recently synthesized DNA strands.[6] One or both of these processes are absent in all cases of FA,[4] meaning that stalled forks are no longer protected. DNA damage then accumulates, and the cells arrest in G_2 phase because of incomplete DNA replication.

But what exactly do the stalled forks need protection from? The main bad guys are nucleases, of which there are 2 kinds: endonucleases such as MRE11 and MUS81 that cut internally to DNA, and exonucleases such as DNA2 or EXO1 that chew up DNA from exposed ends. This is a problem because nuclease-mediated degradation leads to permanent loss of genetic information (which can drive either cell death or cancer-causing mutations, depending on the context of the genes affected). Importantly, short interfering RNAs or chemical inhibition of MRE11 or DNA2 nucleases can significantly reduce the degradation of stalled forks and
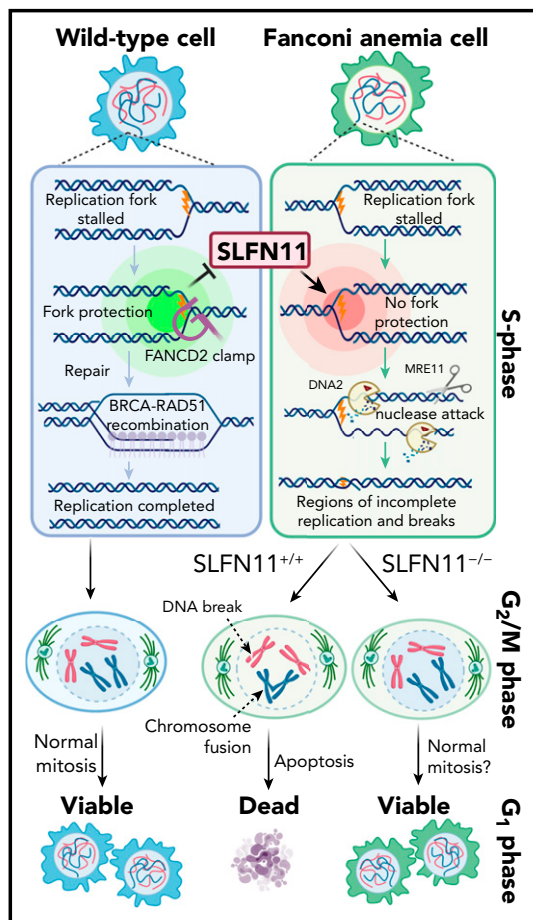
FA cells are deficient in fork protection, which leads to SLFN11-mediated DNA damage and death. During DNA synthesis (S-phase) in wild-type cells, DNA replication forks can be stalled by ICLs or other DNA damage (yellow lightning bolt). In wild-type cells, fork protection is activated by the Fanconi pathway. This includes 2 key steps: formation of a FANCD2 DNA clamp adjacent to the damaged DNA that activates repair, and BRCA-RAD51–mediated recombination that restarts DNA replication. In FA cells, this fork protection is absent. Instead, aberrant RAD51 promotes nuclease attack by DNA2 and MRE11 nucleases. SLFN11 is normally regulated as part of fork protection, but it activates the RAD51-DNA2-MRE11 destruction of forks in Fanconi cells and triggers their G_2/M arrest and/or death. Consequently, SLFN11 deletion in Fanconi cells restores normal mitoses and wild-type levels of viability after DNA damage. Figure created with Biorender.com.

even ameliorate the crosslinker sensitivity of FA cells.[3] In their newly published work, Okamoto et al have shown that at unprotected forks, these nucleases manage to get access to the unprotected DNA with the help of SLFN11.

The *Schlafen* family of genes, named after the German word meaning "to sleep," were originally identified as factors that suppress the growth of thymocytes. Of these, SLFN11 is particularly highly expressed in blood cell lineages and plays a role in native immune, interferon, and T-cell responses during normal development (reviewed in Murai et al[7]). In an unbiased screen of the NCI-60 cancer set, SLFN11 is also the gene whose mutation is most highly correlated with resistance to DNA-damaging chemotherapies.[8]

Okamoto et al used a series of genetic and single-molecule experiments to show that suppression of SLFN11 expression leads to resistance of FANCD2- or FANCA-deficient fibroblasts to cisplatin, mitomycin C, or formaldehyde. They also discovered that SLFN11 deletion reversed the characteristic G_2 arrest and chromosome instability of FA cells. By using single-molecule chromatin fiber techniques, wild-type levels of fork protection in $FANCD2^{-/-}$ $SLFN11^{-/-}$ HAP1 cells was observed. This indicated that SLFN11 normally lets the bad guys in at stalled forks in $FANCD2^{-/-}$ cells. But how does it do this?

All 13 *Schlafen* proteins contain a core slfn box, but at least half also contain a putative DNA unwinding domain of the superfamily 1 helicases. The adenosine triphosphate

(ATP)-dependent unwinding of DNA and RNA structures at DNA damage sites is a critical role of many such helicases. In a final set of experiments, the Okamoto team demonstrated the necessity of a functional ATP-binding site for SLFN11 to facilitate mitomycin sensitivity in FA. This points to a helicase-based mechanism, which, through further experiments, was shown to involve suppression of recombination-associated factors such as RAD51 at stalled forks. A different study showed by various techniques that SLFN11 ATPase activity is also required to open chromatin in the vicinity of stalled forks,[9] which may expose the DNA to nuclease attack. In support of this hypothesis, DNA2 or MRE11 inhibition suppresses the fork degradation of $FANCD2^{-/-}$ cells that overexpress SLFN11. Thus, SLFN11 is a major factor that promotes nuclease attack on unprotected replication forks in FA cells, ultimately causing their death.

So, SLFN11 drives the death of FA cells. This might make it a target for inhibition in suppressing the phenotypes of FA. But unfortunately, this has not proved to be beneficial with other genetic suppression mechanisms observed in vitro in FA. For example, suppressing nonhomologous end-joining factors can rescue the mitomycin C sensitivity of FA cells in culture, but leads to lethality in mice.[10] It remains to be determined whether SLFN11 deletion or inhibition could be similarly problematic, but given that SLFN11 is inactivated in many tumor types and at least 50% of cancer cell lines,[7,9] it is potentially a tumor suppressor. Inactivating tumor suppressors is fraught with danger. Future work should include systematic evaluation of mouse and tumor growth models in addition to examination of blood differentiation rescue phenotypes in a double-mutant *FA* and *SLFN11* background.

FA is a complex disorder that has influenced our understanding of how cancer cells respond to interstrand crosslinking chemotherapies.[4] SLFN11 seems to be another important part of the puzzle. The essential function of SLFN11 in inducing the death of FA cells (and probably most cancer cells, too) means it is likely to be the subject of significant further investigation for treatment of cancer and FA.

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REFERENCES

1. Okamoto Y, Abe M, Mu A, et al. *SLFN11* promotes stalled fork degradation that underlies the phenotype in Fanconi anemia cells. *Blood*. 2021;137(3):336-348.

2. Rosado IV, Langevin F, Crossan GP, Takata M, Patel KJ. Formaldehyde catabolism is essential in cells deficient for the Fanconi anemia DNA-repair pathway. *Nat Struct Mol Biol*. 2011;18(12):1432-1434.

3. Schlacher K, Wu H, Jasin M. A distinct replication fork protection pathway connects Fanconi anemia tumor suppressors to RAD51-BRCA1/2. *Cancer Cell*. 2012;22(1):106-116.

4. Kolinjivadi AM, Crismani W, Ngeow J. Emerging functions of Fanconi anemia genes in replication fork protection pathways. *Hum Mol Genet*. 2020;ddaa087.

5. Tan W, van Twest S, Leis A, et al. Monoubiquitination by the human Fanconi anemia core complex clamps FANCI:FANCD2 on DNA in filamentous arrays. *eLife*. 2020;9: e54128.

6. Daza-Martin M, Starowicz K, Jamshad M, et al. Isomerization of BRCA1-BARD1 promotes replication fork protection. *Nature*. 2019; 571(7766):521-527.

7. Murai J, Thomas A, Miettinen M, Pommier Y. Schlafen 11 (SLFN11), a restriction factor for replicative stress induced by DNA-targeting anti-cancer therapies. *Pharmacol Ther*. 2019; 201:94-102.

8. Sousa FG, Matuo R, Tang SW, et al. Alterations of DNA repair genes in the NCI-60 cell lines and their predictive value for anticancer drug activity. *DNA Repair (Amst)*. 2015;28:107-115.

9. Murai J, Tang SW, Leo E, et al. SLFN11 blocks stressed replication forks independently of ATR. *Mol Cell*. 2018;69(3):371-384.e6.

10. Nie Y, Li Y, Li X, Wilson AF, Pang Q. The non-homologous end-joining activity is required for Fanconi anemia fetal HSC maintenance. *Stem Cell Res Ther*. 2019;10(1):114.
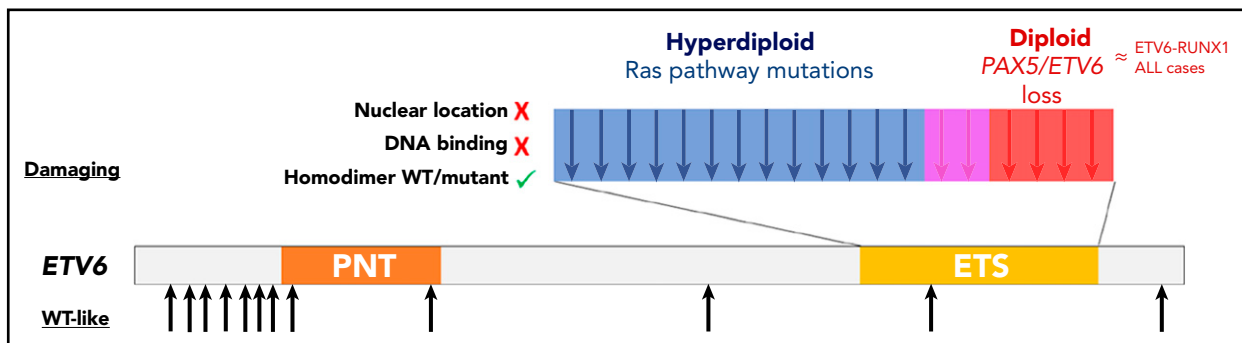

LYMPHOID NEOPLASIA

Germline ETV6 variants: not ALL created equally

Ana Rio-Machin and Jude Fitzgibbon | Queen Mary University of London

In this issue of *Blood*, Nishii et al complete a comprehensive functional assessment of 34 *ETV6* germline variants identified in a previous screen of 4405 patients with pediatric acute lymphoblastic leukemia (ALL).[1] They show that *ETV6* germline variants are not created equally, with 22 of 34 confirmed as damaging and the remainder considered wild-type (WT) like.

Childhood ALL cases with *ETV6* germline mutations were first reported by 3 groups independently in 2015 and constitute a novel leukemia predisposition syndrome that represents 1% of ALL patients.[2-4] In their manuscript, Nishii et al separate 34 germline variants into 2 groups, damaging and WT-like, based on their effect on ETV6 function. Damaging variants are readily distinguishable from WT-like because they preferentially locate to the ETS functional domain and result in reduced ability to bind DNA and lower transcription repressor activity. Critically, damaging variants retain their dimerization potential and behave in a dominant-negative manner, sequestering the remaining WT ETV6 protein to the cytoplasm and disrupting residual function. In contrast, the 12 designated WT-like variants are dispersed across the entirety of the gene and appear to preserve the function of the WT ETV6 protein (see figure) and, unlike their damaging counterparts, are more frequently detected in a non-ALL control population from the gnomAD dataset.

It is interesting that the subsequent trajectory of the disease does not appear to depend directly on these *ETV6* variants, but instead is affected by the nature of the secondary events acquired before the onset of overt disease. Seventy percent of damaging variants reported by Nishii et al were associated with hyperdiploid ALL, and these possessed a distinctive pattern of somatic mutations affecting the *RAS* pathway and differed from the corresponding diploid cases (30%), which acquire *PAX5* mutations or *ETV6* copy number loss (see figure). On closer inspection of the individual variants, it is notable that patients with identical *ETV6* mutations (eg, p.R359X, p.R433H) subsequently develop either diploid or hyperdiploid forms of ALL. Moreover, analysis of families with segregating germline *ETV6* variants reveal an overall 2:1 ratio of ALL to acute myeloid leukemia occurrence,[5] suggesting that the origin of these acquired mutations will instruct the type of subsequent malignancy.

The contribution of secondary genetic events was further supported by transcriptomic analysis in which a comparison of damaging and WT-like ETV6 with 231

Schematic representation of the distribution and characteristics of the ALL-associated *ETV6* germline variants. Each arrow represents a genetic variant: in black, WT-like germline variants; in blue, damaging germline variants associated with hyperdiploid status; in red, with diploid status; and in purple, common to both.