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BCL10 mutations define distinct dependencies guiding precision therapy for DLBCL

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Contributions

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Conflict of Interest

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Abstract

ABC-DLBCLs have unfavorable outcomes and chronic activation of CBM signal amplification complexes that form due to polymerization of BCL10 subunits, which is affected by recurrent somatic mutations in ABC-DLBCLs. Herein, we show that BCL10 mutants fall into at least two functionally distinct classes: missense mutations of the BCL10 CARD domain and truncation of its C-terminal tail. Truncating mutation abrogated a novel motif through which MALT1 inhibits BCL10 polymerization, trapping MALT1 in its activated filament-bound state. CARD missense mutation enhanced BCL10 filament formation; forming glutamine network structures that stabilize BCL10 filaments. Mutant forms of BCL10 were less dependent on upstream CARD11 activation and thus manifested resistance to BTK inhibitors, whereas BCL10 truncating but not CARD mutants were hypersensitive to MALT1 inhibitors. Therefore, BCL10 mutations are potential biomarkers for BTK inhibitor resistance in ABC-DLBCL and further precision can be achieved by selecting therapy based on specific biochemical effects of distinct mutation classes.

Introduction

The CARD11/BCL10/MALT1 complex plays a critical role integrating signaling pathways involved in immunity and inflammation in a broad repertoire of cell types. In B-cells and T-cells, the CBM complex is activated downstream of B-cell receptor (BCR) or T-cell receptor (TCR) signaling and serves to amplify such signals leading to powerful phenotype responses conferred by critical downstream mediates[1,2]. Accordingly, aberrant CBM function has been shown to play critical roles in diseases such as B-cell lymphoma and auto-immunity[3,4]. Upon antigen receptor engagement, the CARD11 subunit is phosphorylated by protein kinase C (PKC), which activates its function by reducing interaction of its auto-inhibitory coiled coil domain to its CARD domain[5–8]. The activated form CARD11 then can interact with BCL10 and facilitate its forming of large macromolecular filaments, providing a large scaffold for binding and activation of MALT1, which is the enzymatic paracaspase subunit of the CBM complex that results in further downstream activation of a variety of effector molecules[9,10].

Like other supramolecular organizing center (SMOC) mediated signaling transduction, such as toll-like receptor (TLR) triggering Myddosome[11,12], RIG-I like receptor sensing intracellular viral RNA and activating mitochondrial antiviral signaling protein (MAVS) filament formation[13], the BCL10 filament formation is also critical for BCR/TCR signaling amplification and robust downstream NF-κB activation[10]. BCL10 is composed of an N-terminal CARD domain, and a long C-terminal unstructured region containing a distal Ser and Thr rich region. Structure guided studies showed that BCL10 filament polymerizes

in a unidirectional manner through CARD-CARD interactions, providing a surface for cooperative binding of MALT1 through its N-terminal Death Domain[10,14]. Upon BCL10 filament binding, MALT1 is immediately dimerized and incorporates TRAF6 to form higher ordered assembly leading to all-or-none activation of downstream pathways including NF-κB and JNK[10,14]. Binding to BCL10 also activates MALT1 paracaspase activity and cleavage of substrate proteins. BCL10 filament formation is dynamic in activated T lymphocytes and precisely regulated by disassembly and degradation through BCL10 K63 polyubiquitination and p62 dependent selective autophagy-lysosomal proteolysis system[15,16]. Hence dynamic BCL10 filament turnover might be critical to precisely tune its effect on downstream signaling pathways such as NF-κB.

Chronic active NF-κB signaling is a hallmark of the highly aggressive activated B cell-like diffuse large B-cell lymphomas (ABC-DLBCLs), due to somatic mutations of BCR and Toll-like receptors (TLR) subunits such as CD79b and MYD88[17–19], as well as activating mutations of CARD11 and amplifications of MALT1[20–22]. Collectively these mutations induce chronic activation of the CBM complex to maintain robust and sustained NF-κB and other downstream pathway activation. The involvement of these signaling pathways in highly aggressive tumors has inspired development of targeted therapies disrupting oncogenic BCR/TLR activity. However, the position where mutations happen in the BCR pathway may be critical for assigning potential precision therapy to patients. For example, mutations in the most upstream BCR proteins like CD79B confer sensitivity to BTK inhibitors, whereas downstream mutations like PLCγ2 and CARD11 confer resistance[23–26]. Hence mechanistic study of oncogenic mutations is beneficial to guide targeted therapy in B cell lymphomas.

Recent genomic sequencing studies in DLBCLs and other lymphomas have revealed recurrent and widely spread somatic mutations of BCL10[27–30]. However, the functionality and mechanism of BCL10 mutations in DLBCL have not been studied. Whereas it is evident how mutations causing constitutive activation of CARD11 or increased abundance of MALT1 might result in enhanced CBM function[20–22], it is not immediately clear how these BCL10 mutations might function. Therefore, we set out to explore the structure and function of BCL10 mutations in DLBCL, identifying distinct classes of mutant proteins with different biochemical effects and distinct impact on response to targeted therapies. These studies have critical implications for selecting targeted therapy agents for lymphoma precision therapy.

Results

BCL10 mutations are genetic drivers and occur in two broad classes.

As a first approach to explore structure-function of BCL10 mutations we merged DLBCL sequencing databases[27,29–32] (n=2255) and identified 75 BCL10 mutant patients. BCL10 contains a structured CARD domain at its N-terminal half that mediates interaction with CARD11 and polymerization of BCL10 into fibrils. The C-terminal region is unstructured and contains serine and threonine residues targeted by post-translational modifications[9,33]. BCL10 mutations affected both regions. Although mutations in the CARD were all missense mutations with a prominent hotspot at Arginine 58, in contrast a majority of those in the

C-terminal region were truncating (nonsense or frameshift) mutations with a number of hotspot residues observed (Fig. 1A and B).

Examining BCL10 mutations in a cohort of patients with rigorous cell of origin and genetic cluster information[29,34], we observed that 51% occurred in ABC-DLBCLs, 31% in unclassifiable cases, and 18% in GCB-DLBCLs (Fig. 1C). However the incidence of BCL10 mutation was highest in the unclassifiable patients, followed by the ABC-DLBCLs (Supplementary Fig. S1A). As reported previously[35] using the LymphGen classification system, most BCL10 mutations were observed in the BN2 class of DLBCLs (Supplementary Fig. S1B), which had the highest incidence of these lesions (35% of patients, Supplementary Fig. S1C). BCL10 expression was also highest among the BN2 cases (Supplementary Fig. S1D).

To determine whether BCL10 somatic mutations were likely to be robust genetic drivers of ABC-DLBCLs, we performed a rigorous genomic co-variate “Fish-hook” analysis[36] controlling for gene size, as well as GC B-cell gene expression profiles, activating promoter histone marks, chromatin accessibility profiles and others. This analysis captured BCL10 as one of the top 10 driver mutations in ABC-DLBCL, along with genes such as MYD88, CD79B, PIM1 and TP53 (FDR<0.01, Fig. 1D). BCL10 mutations were still among the top 15 drivers when considering all DLBCLs (Supplementary Fig. S1E).

Next, to survey whether the different classes of BCL10 mutations had a functional impact on NF-κB signaling, we expressed a panel of CARD and C-terminal mutants, as well as wildtype BCL10 together with an NF-κB luciferase reporter in 293T cells. As expected, WT BCL10 was able to induce NF-κB activity[37,38] (Fig. 1E). Most of the CARD and C-terminal mutations also induced NF-κB activity. However, the hotspot missense mutant $BCL10^{R58Q}$ showed significantly higher NF-κB activity compared to wildtype BCL10, as did the truncating mutants $BCL10^{E140X}$ and $BCL10^{K146Nfs*2}$ (Fig. 1E). Focusing our studies on representative CARD and C-terminal mutations, we showed that this markedly increased NF-κB reporter induction even occurred in ABC-DLBCL cells that already have chronic BCR activation including both MCD (HBL1) and BN2-DLBCL cells (RIVA) (Fig. 1F), also validating that this reporter reacts as expected to disruption of NF-κB signaling (Supplementary Fig. S1F). Hence, both missense and truncating BCL10 mutations yield a significant gain of function effect on NF-κB activation. Using an additional MCD cell line TMD8, we also noted reduction in IκBα abundance in cells expressing $BCL10^{E140X}$ truncating mutant (Supplementary Fig. S1G). Finally, to determine if such findings could be validated in primary human DLBCLs, we performed immunohistochemistry staining of p65 in a set of tissue microarrays containing biopsies from 298 genetically annotated DLBCL patients. We found that BCL10 mutant DLBCLs manifested significantly increased p65 nuclear staining scores compared to BCL10 WT cases (Mann-Whitney $p<0.0001$, Fig. 1G). BCL10 truncating mutations associated with the highest nuclear p65 scores, whereas $BCL10^{R58Q}$ CARD mutants less abundance of nuclear p65 that was still higher than in BCL10 WT cases (Fig. 1H).

BCL10 polymerization is greatly and moderately enhanced respectively by E140X and R58Q mutants.

To determine the mechanism through which BCL10 mutants might confer a biochemical gain of function, we expressed and purified full-length $BCL10^{WT}$, $BCL10^{E140X}$ and $BCL10^{R58Q}$ fused to a 3C protease-cleavable maltose binding protein (MBP) at the N-terminus to keep the proteins in a monomeric state (Fig. 2A). The purified proteins were labeled with Alexa488 through cysteine residues in the unstructured C-terminal region. Spontaneous filament formation was then assessed by confocal fluorescence microscopy as a function of BCL10 concentration 4 h after mixing with the 3C protease to remove the MBP tag. Judging from these images, $BCL10^{WT}$ had a critical concentration of polymerization at ~0.5 μM while $BCL10^{R58Q}$ initiated filament formation at a somewhat lower concentration of ~0.25 μM (Fig. 2B). Strikingly, $BCL10^{E140X}$ started to form filaments even at the lowest concentration tested of 0.1 μM, indicating greatly enhanced ability to polymerize spontaneously (Fig. 2B). Of note, even MBP-fused $BCL10^{E140X}$, in which MBP should suppress filament formation sterically, had a significant polymerized fraction during protein purification; in contrast, we did not observe this phenomenon for $BCL10^{WT}$ or $BCL10^{R58Q}$ (Supplementary Fig. S2A). We further imaged the kinetics of filament formation by these proteins at 1 μM concentration, which is above the critical concentration of polymerization for WT and these mutant BCL10 proteins using time lapse confocal fluorescence microscopy. We found that $BCL10^{E140X}$ was already extensively polymerized at 2 min post-mixing with 3C protease, the earliest time point we could image (Fig. 2C, Supplementary movies 1–3). While filament formation kinetics of $BCL10^{WT}$ and $BCL10^{R58Q}$ were both slower than that of $BCL10^{E140X}$, $BCL10^{R58Q}$ showed more apparent filament formation at 32 min after addition of the 3C protease than $BCL10^{WT}$ (Fig. 2C, Supplementary movies 1–3), suggesting that $BCL10^{E140X}$ and to a lesser extent $BCL10^{R58Q}$ accelerated BCL10 polymerization threshold and kinetics.

Activated CBM complexes manifest as puncta when visualized through confocal microscopy of living cells[20,39,40]. We therefore imaged ABC-DLBCL cells (HBL1) engineered for constitutive expression of FLAG-tagged $BCL10^{WT}$, $BCL10^{E140X}$ and $BCL10^{R58Q}$, respectively. These experiments revealed large, striking aggregates of $BCL10^{E140X}$, in comparison with the much smaller puncta of $BCL10^{WT}$ or $BCL10^{R58Q}$ (Fig. 2D), supporting the in vitro findings using recombinant proteins. These aggregates are reminiscent of the large puncta of an oncogenic, gain-of-function CARD11 mutant[20,41], suggesting that these aggregates represent active CBM complexes.

The R58Q mutant forms filaments with a glutamine ladder, enhanced stability and tendency to bundle.

To better visualize these filaments, we purified 3C protease-treatment induced filaments of $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ by collecting the void fraction (i.e. polymerized BCL10) from a Superose 6 gel filtration column, and imaged them using scanning electron microscopy (EM) (Fig. 3A). Surprisingly, whereas $BCL10^{WT}$ and $BCL10^{E140X}$ formed the classical 10 nM filaments of CARDs[10], $BCL10^{R58Q}$ formed both 10 nM filaments and thicker 20 nM filaments (Fig. 3A). Closer inspection of the 20 nM filaments suggested that

they are bundled 10 nM filaments because these thinner filaments were observed to merge into thicker filaments (Fig. 3A).

To determine if the $BCL10^{R58Q}$ filaments are structurally different from $BCL10^{WT}$ or $BCL10^{E140X}$ filaments and how the bundling occurs, we collected cryo-EM data using an Arctica microscope operating at 200 keV and a K2 electron counting direct detection camera (Supplementary Fig. S3A). We manually selected thin and thick filaments from the cryo-EM micrographs and noted that 2D classification revealed average filaments of similar thickness (Fig. 3B). These data suggested that the association between filaments within the bundle is not specific so that only one thin filament within each thick filament could be aligned and the other filament was averaged out. Thus, the thick filaments consist of randomly bundled thin filaments (Fig. 3B).

Using 3D reconstruction, we determined the cryo-EM structure of the $BCL10^{R58Q}$ filament at 4.6 Å resolution assessed by gold-standard Fourier shell correlation (FSC) (Supplementary Fig. S3B). The overall structure was similar to that of the $BCL10^{WT}$ filament[10] with only the CARD domain ordered (Fig. 3C). Q58 resides in helix 3 (H3) of the six-helical bundle fold of the CARD domain (Fig. 3D). Within the $BCL10^{R58Q}$ filament structure, Q58 localizes near the center and its side chain density is well defined (Fig. 3E), which contrasts with the poor density of the equivalent wild type R58 in the $BCL10^{WT}$ filament structure (Supplementary Fig. S3C). While the resolution of the structure is limited, it is tempting to speculate that Q58 residues at the center of the filament formed stacks of glutamine residues, with direct and possibly water-mediated interactions. Indeed in the center of the filament, we found that Q58 side chain (NE2 atom) of one protomer in the filament forms a potential hydrogen bond with the carbonyl oxygen of T59 of the next protomer in the helical filament (Fig. 3F). To further demonstrate the potential role of the hydrogen bonding network in BCL10 filament assembly in the R58Q mutant, we generated the R58E mutant which does not have the NE2 atom (e.g. Q58 side chain) for hydrogen bond formation, and characterized its biochemical and biophysical properties. We found that there was no enhancement of filament formation for R58E (Supplementary Fig. S3D) and the critical concentration for R58E polymerization is ~ 1 μM was even higher than WT BCL10 (Supplementary Fig. S3E), confirming that the hydrogen bond formed by Q58 is critical for its filament formation.

These Q58-mediated interactions prompted us to ask whether there might be a difference in the stability of the $BCL10^{R58Q}$ filament in comparison to $BCL10^{WT}$ and $BCL10^{E140X}$ filaments. To assess this possibility, we performed thermal melt assays on purified $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ filaments, which revealed that $BCL10^{R58Q}$, and to a lesser degree $BCL10^{E140X}$, yielded more stable filaments, with thermal melting temperatures of 80.8 °C and 78.8 °C respectively, in comparison with 76.6 °C for the $BCL10^{WT}$ (Fig. 3G). Thus, while $BCL10^{E140X}$ shows enhanced polymerization, $BCL10^{R58Q}$ forms more stable filaments, which may explain its tendency to bundle.

Loss of basal MALT1 binding promotes spontaneous polymerization of $BCL10^{E140X}$.

To investigate how the truncation mutants might affect CBM complex formation, we expressed Flag-tagged WT and mutant forms of BCL10 in Raji cells, which lack constitutive

BCR signaling. Performing anti-Flag co-immunoprecipitations, we observed equivalent enrichment for MALT1 in WT and BCL10^{R58Q} as well as in another CARD missense mutant BCL10^{R87Q}. In contrast, there was less binding of MALT1 to BCL10^{E140X}, as well as the similar BCL10^{K146Nfs*2} truncation mutant (Fig. 4A). While BCL10 CARD mutants interacted with CARD11 marginally better than BCL10^{WT}, the C-terminal mutants manifested much greater CARD11 interaction, which is likely due to the increased spontaneous polymerization of truncated BCL10, since BCL10 polymers were shown to enhance interaction with CARD11[42]. The observed weaker recruitment of MALT1 by BCL10 truncation mutants was surprising as previous studies have mapped BCL10 CARD as MALT1 interacting domain (Fig. 4B) shown by mutagenesis and cryo-EM structure of the BCL10-MALT1 filamentous complex[14,38].

MALT1 has multiple domains (Fig. 4C) and in the reported cryo-EM structure, only the MALT1 death domain (DD) was ordered and interacted with the BCL10 CARD (residues 1–115), whereas the MALT1 immunoglobulin-like domains (Ig1-Ig2) and the paracaspase domain were not visible[14]. Given that a previous mapping study suggested that the Ig1-Ig2 domains of MALT1 also interact with BCL10[43], we wondered if there were additional MALT1-binding sites on BCL10 at its largely unstructured C-terminus. We thus divided the C-terminal region into two halves, and found that the second half (residues 165–233), but not the first half (residues 116–164), pulled down the Ig1-Ig2 construct of MALT1 when co-expressed in *E. coli* shown by Coomassie blue-stained SDS-PAGE gel (Fig. 4D). Further truncations of the BCL10 165–233 fragment showed that a construct containing residues 165–208 was sufficient to pull down MALT1 Ig1-Ig2 (Fig. 4E), confirming this second MALT1-binding site (Fig. 4B). Of note, unlike the interaction between BCL10 CARD and MALT1 DD in the filamentous form, the interaction between BCL10 C-terminal region and MALT1 Ig1-Ig2 is monomeric as assessed by gel filtration chromatography of the complex (Supplementary Fig. S4A).

Given that this new MALT1 binding domain is deleted from BCL10^{E140X} we next explored whether there was any impairment in MALT1 recruitment to BCL10 filaments, by generating BCL10^{WT} and BCL10^{E140X} filaments *in vitro* and incubating them with purified, full length MALT1 followed by negative staining EM. These experiments showed equivalent patterns of MALT1 decorating the surface of WT and BCL10^{E140X} filaments, suggesting that MALT1 recruitment was intact in each case (Fig. 4F). However, given that this analysis does not have sufficient resolution to show whether MALT1 distribution on BCL10 polymers was altered, we next collected cryo-EM data on a Titan Krios microscope operating ats 300 keV and equipped with a Falcon II direct electron detector, and determined the cryo-EM structure of BCL10^{E140X} filaments in complex with MALT1 at 4.3 Å resolution. The structure of the BCL10^{E140X} filament with MALT1 was highly similar to that of the BCL10^{WT} filament with MALT1 at 4.9 Å resolution[14], in which the DDs of MALT1 bind the CARD of BCL10 and decorate the outside of the core CARD filament (Fig. 4G–I). The conserved structure confirmed that the truncation did not affect the association of BCL10 filaments with MALT1, but that BCL10^{E140X} is nonetheless defective in interacting with monomeric MALT1.

To further investigate these associations, we performed gel filtration analysis from lysates of ABC-DLBCL cells expressing FLAG-tagged $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$, respectively (Supplementary Fig. S4B). Cell fractionation of these lysates revealed a relatively small proportion of $BCL10^{WT}$ or $BCL10^{R58Q}$ in high molecular weight fractions corresponding to filaments, along with a small fraction of MALT1, whereas most BCL10 and MALT1 proteins were in low molecular weight fractions (Fig. 4J). In marked contrast, $BCL10^{E140X}$ was present at higher abundance in high molecular weight fractions, with corresponding enrichment of MALT1 (Fig. 4J). Reciprocally, there was reduced abundance of $BCL10^{E140X}$ and a notable reduction of MALT1 in the lower molecular weight complexes. Similar findings were observed by performing sucrose gradient experiments (Supplementary Fig. S4C).

The association between enhanced polymerization and lack of MALT1 monomeric interaction of $BCL10^{E140X}$ prompted us to hypothesize that MALT1 binding to the C-terminal region of BCL10 might inhibit BCL10 polymerization. To investigate whether this was the case we incubated Alexa488-labeled, MBP-fused $BCL10^{WT}$, $BCL10^{R58Q}$ or $BCL10^{E140X}$ with increasing concentrations of purified MALT1, treated the reactions with C3 protease to remove the MBP moiety, and monitored polymerization kinetics using fluorescence quenching (Fig. 4K). For $BCL10^{WT}$ and $BCL10^{R58Q}$, increasing doses of MALT1 suppressed BCL10 filament formation in a dose dependent manner. By contrast, there was little suppression of $BCL10^{E140X}$ polymerization by MALT1 at any dose. Taken together these data suggest that $BCL10^{E140X}$, and likely other similar truncation mutations, favor BCL10 polymerization in cells not only by reducing its intrinsic critical concentration threshold (Fig. 2B and 2C), but also by abrogating a novel MALT1 inhibitory effect mediated through interaction with the novel BCL10 C-terminal region binding site. By the same token loss of C-terminal tail binding would increase the pool of MALT1 that is available to bind to BCL10 polymers, suggesting that the end result would be potent enhancement of BCL10 filament formation and MALT1 activity.

Differential activation of MALT1 by $BCL10^{E140X}$ vs $BCL10^{R58Q}$.

Because MALT1 dimerization on BCL10 filaments activates its proteolytic function, we wondered whether skewing of MALT1 cellular pools towards the BCL10 polymer bound state in the $BCL10^{E140X}$ setting might lead to higher cellular levels of MALT1 activity. We therefore examined the effect of BCL10 mutants on MALT1 activity within cells in the absence of basal BCR signaling. For this we performed MALT1 enzymatic reporter assays, using a GloSensor protein construct engineered with a specific MALT1 cleavage site[44] in Raji cells. Raji GloSensor cells were then engineered to express $BCL10^{WT}$, $BCL10^{E140X}$ or $BCL10^{R58Q}$ and we observed significantly greater MALT1 enzymatic activation in $BCL10^{E140X}$ cells compared to either $BCL10^{WT}$ or $BCL10^{R58Q}$ (Supplementary Fig. S4D and S4E). There was also greater MALT1 activity when comparing $BCL10^{R58Q}$ to $BCL10^{WT}$ albeit to lesser extent, consistent with the slightly enhanced polymerization kinetics of $BCL10^{R58Q}$. Finally, we performed similar MALT1 protease reporter assays in ABC-DLBCL cell lines expressing $BCL10^{WT}$, $BCL10^{E140X}$ or $BCL10^{R58Q}$, where there is constitutive activation of signaling to the CBM complex. Again $BCL10^{E140X}$ generally yielded the strongest MALT1 activation (Supplementary Fig. S4F and S4G),

and BCL10^{R58Q} generally yielded greater MALT1 activity than WT BCL10. Collectively, these data indicate that both BCL10^{R58Q} and especially BCL10^{E140X}, through distinct mechanisms, lead to aberrantly increased MALT1 activity.

BCL10^{E140X} confers reduced dependency on CARD11 for CBM activation.

Normally, active CARD11 is required to nucleate the formation of BCL10 filaments[9,10]. However, we wondered whether the requirement for CARD11 might be diminished in ABC-DLBCL cells expressing BCL10^{E140X}, given its greater tendency to polymerize and loss of MALT1 inhibitory interactions. We therefore performed CARD11 shRNA knockdown experiments in isogenic ABC-DLBCL cells expressing BCL10^{WT}, BCL10^{R58Q} and BCL10^{E140X}. CARD11 depletion is known to cause proliferation arrest of ABC-DLBCL cells[20] and accordingly we observed significant growth suppression induced by CARD11 knockdown in the presence of wild type BCL10 (Fig. 5A and Supplementary Fig. S5A–C). However, this effect was significantly blunted in the presence of BCL10^{E140X} and to a lesser extent by BCL10^{R58Q}. To determine how this effect might relate to CBM complex function, we tested the impact of CARD11 knockdown on MALT1 activity using GloSensor reporter assays. MALT1 activity was highly impaired after CARD11 knockdown in the presence of WT BCL10, whereas this effect was completely rescued in BCL10^{E140X} cells and partially rescued by BCL10^{R58Q} (Fig. 5B). CARD11 knockdown also reduced NF-κB reporter activation in BCL10^{WT} ABC-DLBCL cells, an effect that was blunted in CARD11 depleted in ABC-DLBCL cells expressing BCL10^{R58Q} and BCL10^{E140X} (Fig. 5C). Hence an additional perturbation explaining BCL10^{E140X} activation of MALT1 may link to its reduced requirement for CARD11 to induce filament formation, consistent with our data showing markedly greater activity in unstimulated B-cells. BCL10^{R58Q}, which does not polymerize as readily as BCL10^{E140X} and is still inhibited by MALT1 monomers retains a greater degree of CARD11 dependency.

BCL10^{R58Q} and BCL10^{E140X} confer distinct levels of resistance to ibrutinib.

BTK inhibitors have emerged as a precision therapy modality for ABC-DLBCLs[23,45]. However, lymphoma cells with inherent or acquired mutations in activating proteins downstream of BTK (e.g. CARD11 mutations that induce potent MALT1 activation) are often resistant to such treatments[23,26]. Given the distinct functional profiles, CARD11 dependencies, and MALT1 activation effects of BCL10 CARD and truncation mutants, we wondered whether and to what extent they might confer BTK inhibitor resistance. We treated our isogenic ABC-DLBCL cells expressing BCL10^{WT}, BCL10^{R58Q} and BCL10^{E140X} proteins with escalating doses of three chemically distinct covalent BTK inhibitors: ibrutinib, acalabrutinib or zanubrutinib, and tested their proliferation rates using an ATP fluorescence assay after 96 hours of drug exposure. BCL10^{R58Q} conferred at least a modest and often significant reduction in response to these drugs (Fig. 6A–C, Supplementary Fig. S6A). In contrast, BCL10^{E140X} conferred far more dramatic resistance in almost all cases.

All three BTK inhibitors yielded potent and dose dependent suppression of MALT1 activity in BCL10^{WT} ABC-DLBCL cells (Fig. 6D–F, Supplementary Fig. S6B–D). However, isogenic BCL10^{R58Q} and BCL10^{E140X} ABC-DLBCL cells manifested significantly less

impact on MALT1, especially in the case of $BCL10^{E140X}$. Analyzing the further downstream impact of the BTK inhibitors on NF-κB reporter activity revealed significant impairment in $BCL10^{WT}$, which was significantly blunted in the presence of $BCL10^{R58Q}$ and $BCL10^{E140X}$ (Supplementary Fig. S6E–G). Finally, we administered ibrutinib (37mpk, oral gavage, Q.D.) to mice bearing $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ expressing ABC-DLBCL xenografts (Fig. 6G). Ibrutinib yielded the expected growth suppression of $BCL10^{WT}$ ABC-DLBCL tumors but had no significant anti-tumor effect against the two mutant forms, which was most clearly evident in the case $BCL10^{E140X}$ (Fig. 6H–K). Collectively, $BCL10^{R58Q}$ and $BCL10^{E140X}$ confer distinct levels of resistance to BTK inhibition, consistent with their different mechanisms of action and impact on MALT1 activation.

BCL10 truncating mutant lymphomas are hypersensitive to MALT1 protease inhibitor.

The fact that BCL10 mutants drive potent MALT1 activation even in the absence of CARD11 and confer reduced response to ibrutinib led us to hypothesize that these cells might be especially dependent on MALT1 and hence highly responsive to MALT1 inhibitors. To explore this question, we tested the impact of three chemically and mechanistically distinct MALT1 inhibitors against our set of isogenic ABC-DLBCL cells. These included: C3, a potent and specific compound that covalently inactivates the MALT1 catalytic pocket[44]; MLT-748, a reversible allosteric compound that binds MALT1 Trp580 side chain thus to lock protease inactive[46] and JNJ-67690246, an allosteric MALT1 inhibitor (Supplementary Fig. S7A)[47]. JNJ-67690246 potently inhibits MALT1 enzymatic activity (IC50 = 15 nM) in biochemical assays and cytokine secretion of IL6/10 (IC50 = 60 nM) in OCI-Ly3 cellular assays (Supplementary Fig. S7B).

Isogenic $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ ABC-DLBCLs were exposed to increasing concentrations of each of these compounds for 96 h, revealing striking differences in the response profiles of $BCL10^{E140X}$ vs $BCL10^{WT}$ and $BCL10^{R58Q}$ (Fig. 7A–C, Supplementary Fig. S7C). Both WT and $BCL10^{R58Q}$ were generally sensitive to the allosteric inhibitors, whereas $BCL10^{R58Q}$ cells were less sensitive than WT to C3. In marked contrast, $BCL10^{E140X}$ manifested significantly greater response to all three MALT1 inhibitors. This differential effect was not due to variation in the degree of MALT1 inhibition, since MALT1 activity was equivalently suppressed by all three drugs in $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ ABC-DLBCL cells (Fig. 7D–F, Supplementary Fig. S7D–F). Analysis of NF-κB reporter activity showed significantly greater impairment in MALT1 inhibitor treated $BCL10^{E140X}$ cells as compared to either $BCL10^{WT}$ or $BCL10^{R58Q}$ (Supplementary Fig. S7G–I), with the latter even showing less impairment of the NF-κB activity than in WT cells, suggesting that some other pathway may be maintaining NF-κB and hence conferring less dependency on MALT1 than in $BCL10^{E140X}$ cells. Finally, we treated $BCL10^{WT}$, $BCL10^{R58Q}$ and $BCL10^{E140X}$ ABC-DLBCL xenografts with JNJ-67690246 *in vivo*, using the OCI-Ly10 cell line which is generally less sensitive to MALT1 inhibition, for greater stringency (Fig. 7A–C, G). We observed significant reduction in growth of $BCL10^{E140X}$ but not $BCL10^{WT}$ or $BCL10^{R58Q}$ lymphomas (Fig. 7H–K), confirming their increased dependency on MALT1 activity and the potential for MALT1 inhibitors to be most useful for patients bearing such mutations.

Discussion

Herein, we show that BCL10, one of the most frequently mutated genes in DLBCL, is a bona fide genetic driver of lymphomagenesis. Importantly, our structure-function studies reveal that these mutations occur in at least two biochemically distinct classes: missense mutations of the CARD domain and truncation mutations of the C-terminal tail. These classes of mutations seem to affect distinct aspects of BCL10 functionality and lead to biologically distinct outcomes as indicated by their differential downstream effects on MALT1 and NF-κB signaling as well as vulnerability to targeted therapies. Many of the BCL10 truncating mutations cluster between AA 135 to 174, and representatives of these mutations manifested the most powerful activation of NF-κB activity. BCL10 truncation mutants such as $BCL10^{E140X}$ manifested a striking increase in its ability to polymerize into its filamentous form, accompanied by potent activation of MALT1 protease activity. This tendency to polymerize, indicated for example by its lower critical concentration threshold may help to explain the reduced CARD11 dependency of lymphoma cells expressing $BCL10^{E140X}$, and is consistent with previous studies showing that BCL10 CARD domain alone can undergo spontaneous polymerization in vitro[9,48]. Although it is generally understood that CARD11 serves to nucleate BCL10 polymerization, it has been suggested that CARD11 association with BCL10 filaments is further stabilized by nascent helical BCL10 polymers[42]. This may explain why we observed increased association of BCL10 and CARD11 in lymphoma cells expressing $BCL10^{E140X}$, given its greater tendency to polymerize, while at the same time being consistent with its reduced requirement for CARD11 to induce filament formation and reduced biological dependency on CARD11 in $BCL10^{E140X}$ expressing DLBCL cells.

Binding of the MALT1 death domain to BCL10 was unperturbed in filaments composed of $BCL10^{E140X}$, which is not surprising since this molecular association is mediated through the BCL10 CARD domain and proximal regions that are not affected by truncation mutation[14,43]. Yet Co-IP experiments paradoxically indicated reduced interaction between BCL10 and MALT1. This prompted us to examine other modes of BCL10-MALT1 association, leading to the identification of a novel direct interaction site between MALT1 Ig1-Ig2 region and BCL10 AAs 165 to 208, a region that is lost in a majority of truncation mutants except for a cluster deleting the extreme C-terminal Ser/Thr rich tail. Importantly, we show that MALT1 impairs BCL10 polymerization through this interaction surface, thus constituting a novel CBM negative regulatory mechanism preventing spurious polymerization of BCL10. This in turn likely explains the dramatically increased filament formation by $BCL10^{E140X}$ in vitro, and its greatly enhanced ability to recruit MALT1 into the polymerized CBM complex, given that loss of monomeric BCL10 and MALT1 binding would increase the pool of MALT1 to associate with filaments. These events occur due to the presence of $BCL10^{E140X}$ thus appear to constitute a positive feedback loop that ultimately causes potent MALT1 protease activation and biological dependency on MALT1 catalytic function. The more distal set of C-terminal truncating mutations such as Q208X, L209X and L225X retain the MALT1 Ig1-Ig2 interacting region and hence would not be expected to escape from this MALT1 inhibitory binding. Accordingly, when expressed in cells, they did not induce greater NF-κB activity than WT BCL10, suggesting that there may

be additional ways in which BCL10 function could be perturbed, perhaps due to specific loss of certain as of yet undiscovered post-translational modifications. Interestingly, L225X produces a truncated form of BCL10 similar to the MALT1 protease dependent cleavage form of BCL10 R228X observed in activated T-cells as well as in ABC-DLBCL cells with chronic active BCR signaling[49,50] and showed similar functional effects to WT BCL10 overexpression in NF-κB reporter assays, perhaps due to retaining both intact MALT1 biding sites. Notably, the cleaved BCL10 R228X form was shown to mediate migratory function in T-cells[49], pointing to the need for further studies of these BCL10 mutations. BCL10 truncating mutations, translocation and amplification were also shown to occur in marginal zone lymphomas including MALT lymphomas[51–54]. However, the impact of BCL10 mutations in MZL has not been explored and warrants further study.

In contrast, missense mutations of the BCL10 CARD domain seem to have distinct functional effects. Many of the BCL10 residues affected by mutations (e.g. R58, K63) are localized within the core of BCL10 helical structures where they make important intrastrand (Type III) and interstrand (Type I) interactions that are critical for filament formation[10,42]. Along these lines an R53Q mutation affecting type III interactions might be predicted to disrupt intrastrand interactions and caused a severe defect in BCL10 CARD domain polymerization. This was however proven to be incorrect, and the sole CARD domain hotspot mutant residue Q58 engages in a novel form of type III interaction resulting in apparent formation of a hydrogen bonded glutamine network. The consequence is a shift in BCL10 polymerization kinetics, favoring the polymerized state but without dramatically altering binding to CARD11 or MALT1 and yielding a more modest gain of function phenotype. Missense mutations such as K63Q might functionally resemble R58Q since they also locate at the central core of the BCL10 CARD filament and have positively charged residues switched to glutamine to potentially enhance, rather than disrupt, interactions.

These biochemical features of the $BCL10^{R58Q}$ mutant result in a hypomorphic phenotype compared to truncation mutant where they induced less potent MALT1 and NF-κB activation than $BCL10^{E140X}$. However, our results also suggest that $BCL10^{R58Q}$ may engage in additional gain of function effects. This is suggested by the fact that cells expressing $BCL10^{R58Q}$ seemed relatively resistant to loss of NF-κB activity upon exposure to MALT1 inhibitors as compared to that on BCL10-WT or $BCL10^{E140X}$, whereas in contrast, reduction in NF-κB activity in response to the upstream BTK inhibitors was similar between $BCL10^{R58Q}$ and $BCL10^{E140X}$. These data prompt us to hypothesize that other functions may derive from the stability or bundling of R58Q filament. For example, more stable BCL10 filaments might enhance MALT1 recruitment and activation of TRAF6, which could partially support NF-κB independently from the MALT1 proteolytic function[10], or activate NF-κB through other alternative means such as linear ubiquitylation (LUBAC) associated mechanisms[55,56]. Engagement of these or other MALT1 paracaspase independent biochemical effects would be consistent with $BCL10^{R58Q}$ DLBCL cells retaining greater dependency on CARD11 and hence upstream signaling and responsiveness to BTKi.

Overall, our data spotlight the complexities involved in developing precision therapies for DLBCLs and other tumors. The identification of chronic active BCR signaling as a characteristic of ABC-DLBCLs has led to intense efforts to integrate BTKi into multi-

modality regimens. However to date, it is clear that many patients still do not benefit from the addition of such compounds. Our data point to biochemical mechanisms that might help to explain this, as exemplified most clearly by the BTKi resistance conferred by BCL10 truncation mutants and this suggests that such patients should not be treated with BTKi containing regimens. Instead, patients with BCL10 truncation mutations would likely best be served by incorporating MALT1 inhibitors. This concept is feasible since several MALT1 inhibitors are already in clinical trials. Although these findings could also be relevant to acquired BTK inhibitor resistance[25,26], as of yet BCL10 mutations have not been identified in this setting. Similar considerations may apply to other (but not all) mutations downstream of BTK, as exemplified by the case of CARD11 coiled-coil domain mutants, which induce resistance to BTKi but not MALT1i. However, the fact that BCL10 CARD domain mutations may still retain BTKi responsiveness further underlines the need for rigorous study of signaling pathway mutations such as these, and perhaps eventually the need for targeted sequencing studies to provide a precision therapy “map” of these tumors allowing selection (or even combination) of the targeted therapies (e.g. BTKi vs MALT1i) appropriate to their specific signaling scenarios.

Materials and Methods

Cell culture

Raji, HBL1, TMD8 and RIVA were cultured in RPMI supplemented with 10% FBS, 2 mM L-glutamine and 10 mM HEPES. OCI-Ly10 was cultured in Iscove’s medium supplemented with 20% FBS, 2 mM L-glutamine. 293T was cultured in Dulbecco’s Modified Eagle Medium with 10% FBS. All cell lines were authenticated by University of Arizona Genetic Core, grown in presence of 1% penicillin G and streptomycin and at 37°C in a humidified atmosphere of 5% CO_2. HBL1 and RIVA was obtained from Jose A. Martinez-Climent (Universidad de Navarra, Pamplona, Spain); TMD8 was obtained from Louis M. Staudt (National Cancer Institute, Bethesda, Maryland, USA); OCI-Ly10 cell lines were obtained from the Ontario Cancer Institute (OCI).

Virus production and transduction

Lentiviruses were produced in 293T cells by co-transfecting shRNA (short hairpin sequences are: shNonTargeting: CAACAAGATGAAGAGCACCAA; shCARD11#1: GGACGACAACTACAACTTAGC; shCARD11#3: TGGTCAAGAAGCTGACGATTC) or overexpression vectors with packaging vectors psPax2 (Addgene#12260, RRID: Addgene_12260) and psMD2.g (Addgene#12259, RRID: Addgene_12259) at the 4:3:1 ratio in serum free media. The supernatant containing virus particles were harvested 48 h and 72 h after transfection, filtered through 0.45 μm filter and then concentrated with PEG-it according to manufacturer’s instructions (LV825A-1, System Biosciences). Virus was resuspended with PBS containing 25 μM HEPES and added to cells for overnight infection. Cells were selected 24 h post transfection by adding puromycin (Sigma), blasticidin (Invivogen) or G418 (Life Technologies) for at least 48 h.

Xenograft

All mice experiments were approved by Institutional Animal Care & Use Committee (IACUC) at Weill Cornell Medicine and were performed following the IACUC guidelines. Eight to ten weeks of female NOD.Cg-prkdc^{scid} Il2rg^{tm1Wjl}/SzJ (NSG, RRID: IMSR_JAX: 005557) mice were obtained from The Research Animal Resource Center (RARC) at Weill Cornell Medicine. 5×10^6 HBL1 or 10^7 million OCI-Ly10 and their derived engineered cells were resuspended with PBS/Matrigel (1:1) and subcutaneously injected to the right flank of mice. Treatments were started when tumor volume reached an average of 100 mm^3. Ibrutinib was prepared in corn oil with 10% (v/v) DMSO or 0.5% methylcellulose in water and administrated p.o. with 25 or 37.5 mg/kg once per day. JNJ-67690246 was prepared in PEG400 with 10% (w/v) PVPVA64 and administrated p.o. with 100 mg/kg twice per day. Tumor volume was monitored 2~3 times/week with digital caliper and calculated using the following formula: smallest diameter^2 × largest diameter × 0.5.

Growth Inhibition Assay

DLBCL cell lines were cultured in exponential condition and the cell growth was determined by CellTiter Glo (Promega). 3000–5000 cells were seeded and cultured in each well of 384 well plate for 96 h and treated with compounds every 48 h. Luminescence was read at the endpoint with the Synergy NEO microplate reader (BioTek). The value of compound treated cells was normalized to their vehicle treated controls and then used to calculate IC50 in GraphPad Prism (RRID:SCR_002798).

NF-κB Reporter Assay

For NF-κB reporter assay in 293T, the plasmids expressing different BCL10 mutations were transiently transfected into 293T cells using Lipofectamine (Invitrogen). Renilla luciferase plasmid was co-transfected as an internal control. 24 h after transfection, cells were collected and luciferase activities were measured in Synergy NEO microplate reader (BioTek) with the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions and normalized to Renilla luciferase activity.

To generate stable NF-κB reporter cells, the lentivirus expressing 3xNF-κB response element followed by a luciferase firefly was made and infect the parental cells. Puromycin was then added for antibiotic selection 24 h after infection. Reporter cells were further validated by BTK inhibitor and MALT1 protease inhibitor treatment and PMA/IO stimulation. NF-κB reporter cells expressing BCL10 were generated by infecting reporter cells with different lentiviral BCL10 isoforms (co-expressing GFP), followed by sorting out GFP+ cells. Stable NF-κB reporter cells were harvested at the indicated conditions and lysed with 1x passive lysis buffer at room temperature for 20 min. The lysate was briefly centrifuged and the supernatant was collected for luciferase activity. All the assays were presented as mean ± SEM of three independent experiments.

MALT1 GloSensor Assay

The generation of Raji MALT1 GloSensor reporter cell has been previously described[44]. All other GloSensor reporter cells were generated by infecting parental cells with lentiviral MALT1-GloSensor (pLex306 backbone), followed by antibiotic (blasticidin) selection. All

derived GloSensor cells were further validated by MALT1 protease inhibitor treatment and PMA/IO stimulation.

Immunoprecipitation

10^8 lymphoma cells were collected, washed with cold PBS and resuspended with lysis buffer (1% NP40, 10% glycerol, 150mM NaCl, 20 mM Tris-HCL pH 7.5, and freshly added protease inhibitors). The lysates were centrifuged at 15,000g, 4°C for 15min and the supernatant was then collected and incubated with 50 μL equilibrated anti-Flag magnetic beads (Sigma-Aldrich Cat# M8823, RRID:AB_2637089) at 4°C for 3 h. The beads were washed 3 times with lysis buffer and followed by 3 times washing with the lysis buffer without NP40. SDS loading buffer without non-reducing reagent was added and boiled at 95°C for 5 min. The elution was added for β-mercaptoethanol (final 10%), and ready to run western blot after boiling at 95°C for 5 min.

Western Blotting

Whole cell lysates extracted with RIPA buffer or IP elution were separated by SDS-PAGE gels and followed by transferring to PVDF membranes. Membranes were incubated with indicated primary antibodies: anti-Flag (Sigma-Aldrich Cat# F3165, RRID:AB_259529), anti-MALT1 (Santa Cruz Biotechnology Cat# sc-46677, RRID:AB_627909), anti-CARD11 (Abcam Cat# ab113409, RRID:AB_10861854), anti-β-Actin (AC-15, Sigma-Aldrich), and then mouse/rabbit peroxidase-conjugated secondary antibodies (Cell Signaling Technology). Protein intensity was detected with enhanced chemiluminescence using ChemiDoc imaging system (Bio Rad).

Driver mutation analysis

The Driver mutation analysis is performed using Fishhook (https://github.com/mskilab/fishHook) on a total of 243 ABC-DLBCL cases from NCI cohort. Fishhook is a model built with mutational calls, a set of hypothesis intervals, eligible genomic ranges and a set of genomic covariates that identifies the depletion and enrichment of genomic interval statistically. The model used a gamma-Poisson regression to implement the maximum likelihood approximation with consideration of user assigned covariates and expected mutation density to the hypothesis. With this approach, the model helps us to identify enriched mutations with consideration like chromatin features, sequence context composition and gene expression.

Eligible region is defined using genecode v19 and fractional coverage of hg19 positions provided by Agilent exome coverage. We also fed the model covariates that defines B cell specific transcriptional states and chromatin state information for the model. The covariates of ABC specific transcriptional states are generated by number of the overlap between the TSS site of genes TPM >2 in the half the ABC-DLBCL cases from the same NCI cohort within 10kb the eligible regions. The covariates of B-cell specific chromatin states are generated by number of the overlap between H3K27Ac Peaks that previously reported in the B cell within 100kb of the eligible regions and the ATAC peaks of the B cells within 10kb of the eligible. A total of 3 covariates was fed to the fishhook model. Here we noted genes of FDR < 0.05 and BCL10 have an FDR of $1.4e^{-10}$. QQ-plot is plotted by pairing observed

−log 10 transformed quantiles of observed P values (y-axis) with their corresponding −log 10 transformed quantiles from the uniform distribution (x-axis).

Biochemical evaluation of MALT1 protease activity

MALT1 protease activity was assessed in vitro using full-length MALT1 protein (Strep-MALT1(1-824)-His) purified from baculovirus-infected insect cells. The tetrapeptide LRSR is coupled to 7-amino-4-methylcoumarin (AMC) and provides a quenched, fluorescent substrate for the MALT1 protease (SM Biochemicals). Cleavage of AMC from the arginine residue results in an increase in coumarin fluorescence measured at 460 nM (excitation 355 nM). Diluted compounds were pre-incubated with MALT1 enzyme for 50 minutes at room temperature (RT). Substrate was added subsequently and the reaction was then incubated for 4 h at RT, after which fluorescence was measured.

IL-6/10 secretion assay using DLBCL cell line

Secretion of the IL-6 and IL-10 cytokines by OCI-Ly3 ABC-DLBCL cells was measured using a Mesoscale assay (MSD). MALT1 inhibition results in a decrease of IL-6/10 secretion. OCI-Ly3 cells were treated with diluted compounds for 24 h at 37°C and 5% CO2. After 24 h of incubation, 50 μL of the supernatant was transferred to an MSD plate (V-Plex Proinflammation Panel 1 [human] kit) and incubated for 2 h at RT followed by a 2 h incubation with IL-6/10 antibody solution. Plates were read on a SECTOR imager.

Protein Expression and Purification

All constructs of BCL10, MALT1 are from human sequences. Full-length WT and mutant BCL10 constructs with N-terminal MBP tag were generated in vector pDB-His-MBP with a 3C protease site between MBP and BCL10. Full length His-tagged MALT1 cloned into pET29b was purchased from Addgene (RRID:Addgene_48968) and was expressed in E.coli.

All proteins were purified by either Ni-NTA resin (Qiagen) or Amylose resin followed by gel filtration chromatography (Superdex 200 10/300 GL, GE Healthcare). BCL10 FL and mutant filaments were purified by MBP affinity column in binding buffer containing 25 mM Tris at pH 7.5, 300 mM NaCl, 1 mM TCEP, followed by Superdex 200 gel filtration chromatography in buffer containing 20 mM Tris at pH 7.5, 150 mM NaCl and 1mM TCEP, resulted in isolation of a monomeric fraction of BCL10. Then monomeric MBP-BCL10 was cleaved by 3C protease and incubated at RT for 2 hours in order to allow filaments formation. This step was followed by another Superdex 200 gel filtration chromatography and BCL10 filaments were isolated at the void peak for structure determination and thermostability assay. Isolation of BCL10 (1–140)-MALT1 complex was performed in a similar way in which BCL10 and MALT1 were purified separately. BCL10 pre-formed filaments after 3C cleavage were added mixed together with FL MALT1 to form a complex.

Negative Stained Electron Microscopy

Copper grids coated with layers of plastic and thin carbon film were glow-charged before 5 μl of purified complexes were applied. Samples were left on the grids for 1 minute followed by negative staining with 1% uranyl formate for 30 seconds and air dried. *In vitro* BCL10

WT and mutants, BCL10/MALT1 and CBM were imaged with JEOL 1200EX or Tecnai G^2 Spirit BioTWIN at Harvard Medical School EM facility operating at 80 keV.

Cryo-Electron Microscopy (Cryo-EM) Data Collection

Cryo grids for BCL10 R58Q and BCL10 E140X/MALT1 filaments were prepared by applying 3μl of protein sample on a c-flat (1.2/1.3) 300 mesh grids. Grids were plunged by using vitrobot (FEI) at 4°c with 3 sec blotting and force 4. For BCL10 R58Q data collection, 3439 movies were collected at super resolution mode with using Arctica microscope at UMASS facility, operated at 200kv facility with k2 camera. The movies were collected automatically using SerialEM data collection at a nominal magnification of 36,000 and a pixel size of 0.435 Å, with a total dose of 38 e/Å2 which was fractionated into 40 movie frames, with defocus range of −1 −2.5 μm. For BCL10 E140X-MALT1 collection, 700 movies were collected at super resolution mode with using 300 kV FEI Titan Krios microscope equipped with FEI Falcon II detector at PNCC cryo-EM facility, operated at 300kv with Falcon3 camera. The movies were collected automatically using SerialEM data collection at a nominal magnification of 47,000 and a pixel size of 0.4 Å, with a total dose of 55 e/Å2 which was fractionated into 40 movie frames.

Cryo Electron Data Processing

For helical reconstruction of BCL10 R58Q and BCL10 E140X/MALT1, Motioncor2 was used for drift correction and Micrographs were CTF corrected by using CTFFIND4. Data was processed with using Relion (3.1). The resolutions of the reconstruction were determined by FSC to 4.6 Å and 4.3 Å, respectively. Model building was performed in program Coot36. Refinement was performed against the using Phenix refine50. Structural presentations were generated using Pymol (DeLano Scientific) and Chimera[57].

Confocal Imaging

Time lapsed movies of full length labeled Alexa488-BCL10 FL, BCL10 R58Q and BCL10 E140X were recorded with using Nikon spinning disk confocal microscope at Harvard Micron facility for periods of 30 min - 1 hr with 1 minute interval, with using x 100 objective. 3C was added at a sub-molar ratio to allow MBP cleavage to occur within 2–3 minutes in order to provide ample time for setting up the microscope and starting the recording.

For critical concentration determination, labeled Alexa488–BCL10 FL, R58Q and E140X filaments were formed at increasing concentrations ranging between 0.1 μM - 1 μM. 4 h after cleavage with 3C and incubation at RT, samples were placed on a 35 mm bottom glass dish and imaged with using spinning disk confocal microscope with using x 100 objective.

Fluorescence Quenching Assay

Purified full length MBP-BCL10, BCL10 R58Q and BCL10 E140X were mixed with 5-fold molar excess of Alexa- 488-C5-maleimide (Invitrogen) and incubated at 4 °C temperature for O/N. Gel filtration chromatography (Superdex 200, GE Healthcare) was used to remove free dyes. Fluorescence polarization assay was performed at 18 °C in buffer containing 20 mM Tris at pH 7.5, 150 mM NaCl, and 0.5 mM TCEP and in 20 μl volume. 3 μM of labeled

MBP- BCL10 were cleaved with 3C in the presence of increasing amount of MALT1. The fluorescence quenching was measured right after 3C addition for 2 h by using NEO plate reader (Biotek) using excitation/emission wavelengths of 495 nM/519 nM.

Protein Stability

Purified BCL10 FL, R58Q and E140X filaments purified from the void peak of Superdex200 were mixed with 1-fold protein thermal shift dye (Thermo Fisher Scientific). Thermal scanning (25 to 95 °C at 1 °C/min) was performed and melting curves were recorded on a StepOne RT-PCR machine. Data analysis was done by Protein Thermal Shift™ Software (Thermo Fisher Scientific).

IF

HBL1 cells cultured in fresh media were mixed in 1:1 ratio with cytospin. Cells were spined at 800×g for 5 min. Cell pellets were resuspended with cytospin and plated on CELLview 4-compartment dishes (Greiner Bio-One). Cells were left at RT o/n and were fixed with 100% cold methanol for 5 minutes at −20 °C, followed by cell permeabilization with 0.1% Triton X-100 in PBS-Tween (PBST) for 10 minutes. Cells were incubated with blocking buffer containing 3% BSA for 3 h, in order to minimize non-specific binding. After blocking, Cells were incubated overnight at 4 °C with FLAG primary antibody (Sigma-Aldrich Cat# F1804, RRID:AB_262044). After incubation, cells were washed with PBST 3 times and incubated with AlexaFluor488-conjugated anti-mouse IgG (Abcam Cat# ab150113, RRID:AB_2576208) for 1 h at room temperature. After incubation, cells were washed with PBS and then stained with Hoechst for 10 minutes (1:500, Immunochemistry Technologies, Cat# 639). Cells were imaged using spinning disk confocal microscope with using x 100 objective.

IHC staining of p65

Formalin-fixed paraffin-embedded (FFPE) tissue sections of 4 μm thickness were cut from a tissue microarray composed of duplicate 0.6 mm cores from 298 cases of de novo DLBCL. Slides were processed using standard immunohistochemistry protocols and stained with an antibody against NF-κB p65 (Cell Signaling Technology Cat# 8242, RRID:AB_10859369, 1:500 dilution). Appropriate staining was verified in sections of benign tonsil, heart and liver. Stained slides were assessed by an expert hematopathologist for nuclear expression of p65 in DLBCL tumor cells, scored as a percentage of tumor cell nuclei.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The cryo-EM structures have been deposited in the Electron Microscopy Data Bank (EMDB) with accession numbers EMD-27095 (BCL10 CARD R58Q) and EMD-27100 (BCL10 E140X-MALT1 DD). The atomic coordinates have been deposited in the Protein Data Bank (PDB) with accession numbers 8CZD (BCL10 CARD R58Q) and 8CZO (BCL10 E140X-MALT1 DD). All other data are available from the corresponding authors with reasonable request.

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Statement of Significance

ABC-DLBCLs feature frequent mutations of signaling mediators that converge on CARD11-BCL10-MALT1 complex. We use structure-function approaches to reveal that BCL10 mutations fall into two distinct biochemical classes. Both classes confer resistance to BTK inhibitors, whereas BCL10 truncations confer hyper-responsiveness to MALT1 inhibitors providing a roadmap for precision therapies in ABC-DLBCLs.

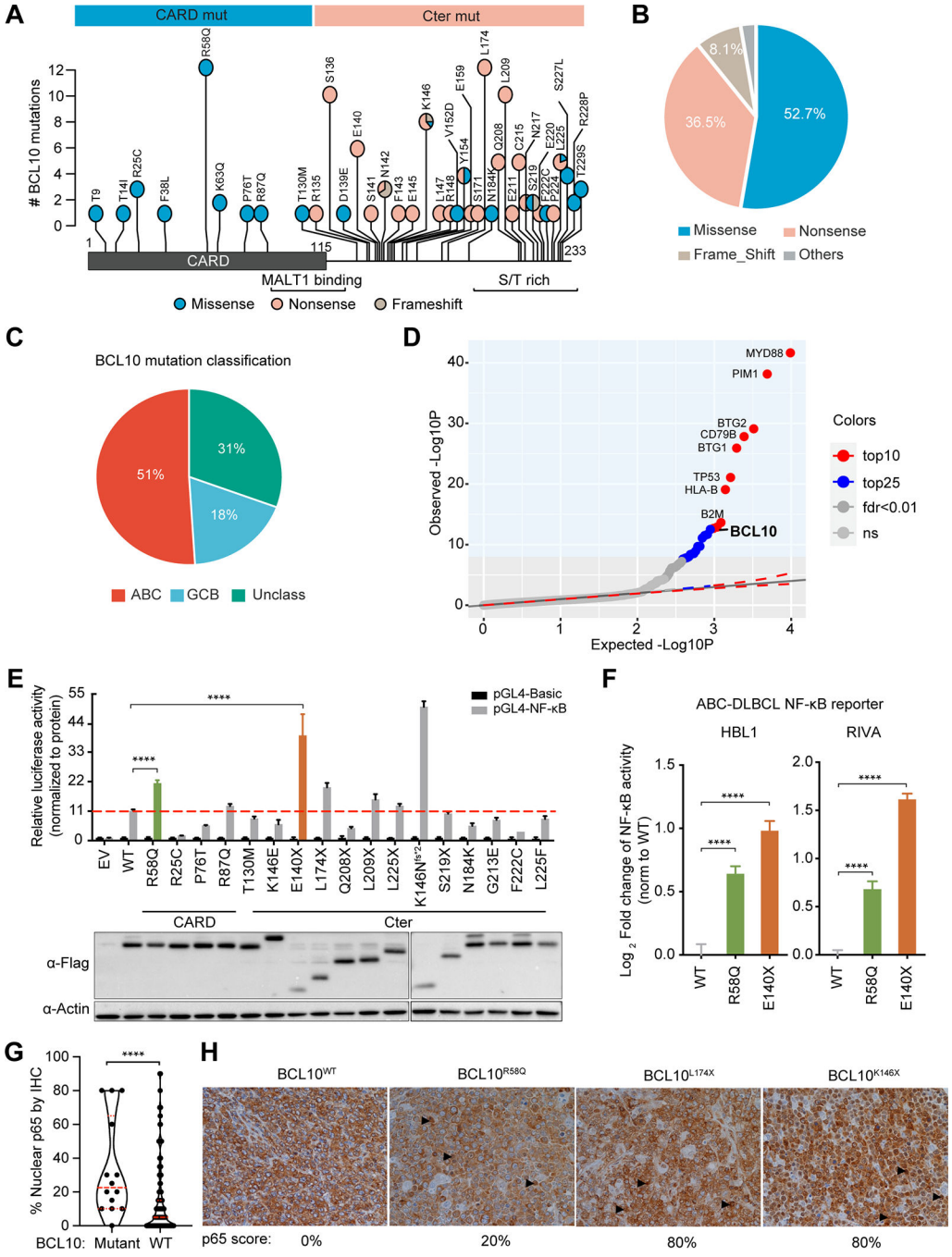

Figure 1 - Characterization of human BCL10 mutations in DLBCL.

A. BCL10 protein domain and the locations of all BCL10 mutations in DLBCL identified and reported from literature and open database.

B. Proportion of mutation types among BCL10 mutant patients.

C. Cell of origin classification of BCL10 mutant DLBCL patients.

D. Quantile-quantile plot showing the p-values for BCL10 SNVs across 243 ABC-DLBCL patients.

E. NF-κB activity measured by NF-κB-RE-luciferase reporter 24 h post transfection of different BCL10 mutations into 293T cells. Luciferase activity was normalized to expression level of each mutation. EV, empty vector. β-Actin was used as internal control. ****p<0.0001.

F. NF-κB reporter activity in lymphoma cells expressing BCL10 mutations. ****p<0.0001.

G. Statistical comparison of nuclear p65 staining scores between BCL10 WT and mutant tumors in TMA for DLBCL patient (n=298). ****Mann-Whitney p<0.0001.

H. Representative images of p65 immunohistochemistry staining in BCL10 WT and mutant DLBCLs in (G). Images were taken under magnification of 400x. Black arrowheads point to examples of p65 nuclear staining.

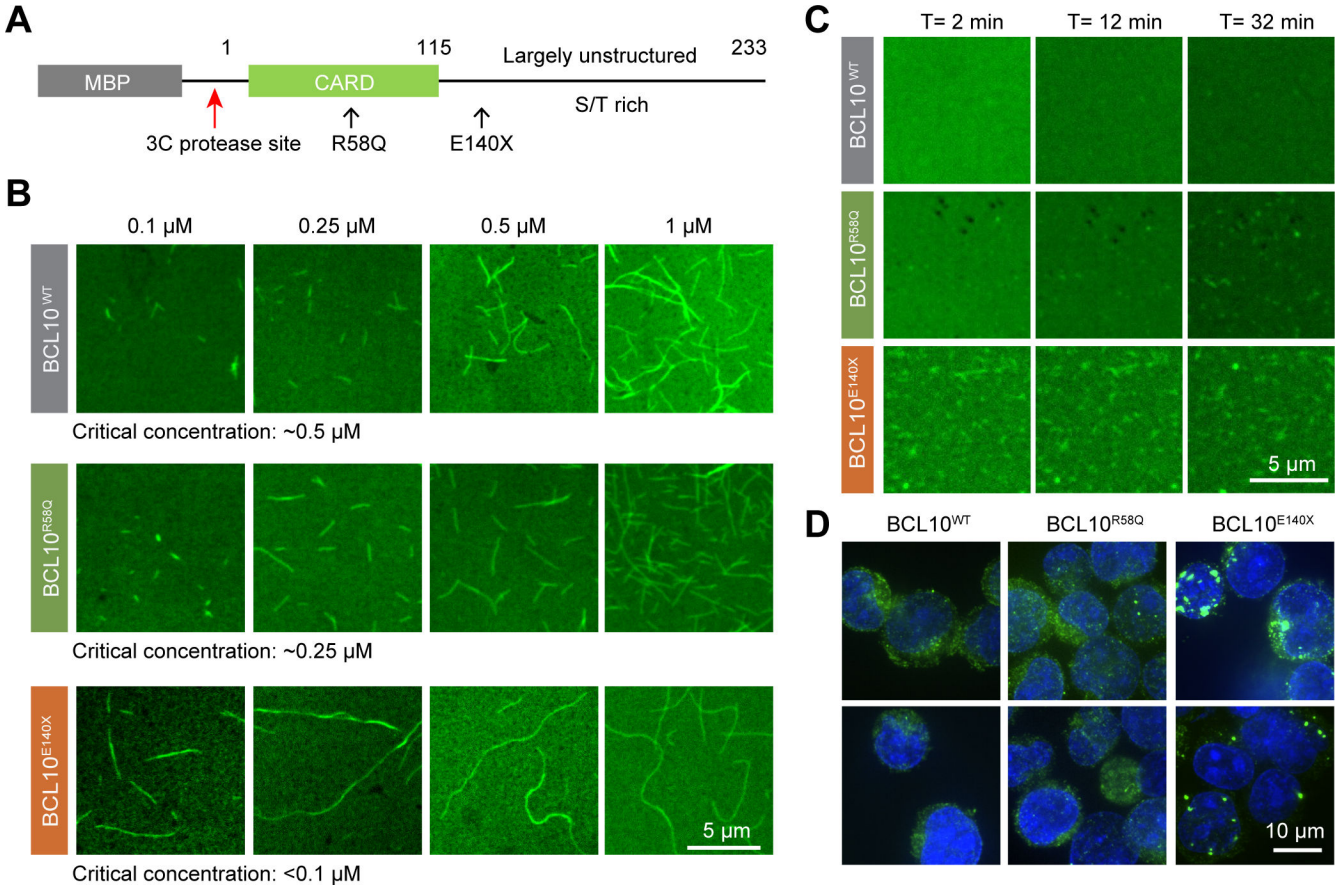

Figure 2 - Representative missense and truncating mutant BCL10 enabled faster polarization rate.

A. Domain organization of MBP-BCL10 construct.

B. Critical concentration determination of WT, E140X and R58Q based on confocal images of BCL10 filaments formed at concentration range between 0.1 μM - 1 μM, imaged 4 h after MBP cleavage. Scale bar 5 μm.

C. Confocal time lapse of WT, E140X and R58Q imaged at 1 μM for 30 min. E140X exhibits the fastest polymerization rate in comparison to WT and R58Q. Scale bar 5 μm.

D. Confocal images of HBL1 cells stably expressing Flag tagged WT, E140X and R58Q, visualized by IF for BCL10 and DNA (Hoechst dye, blue).

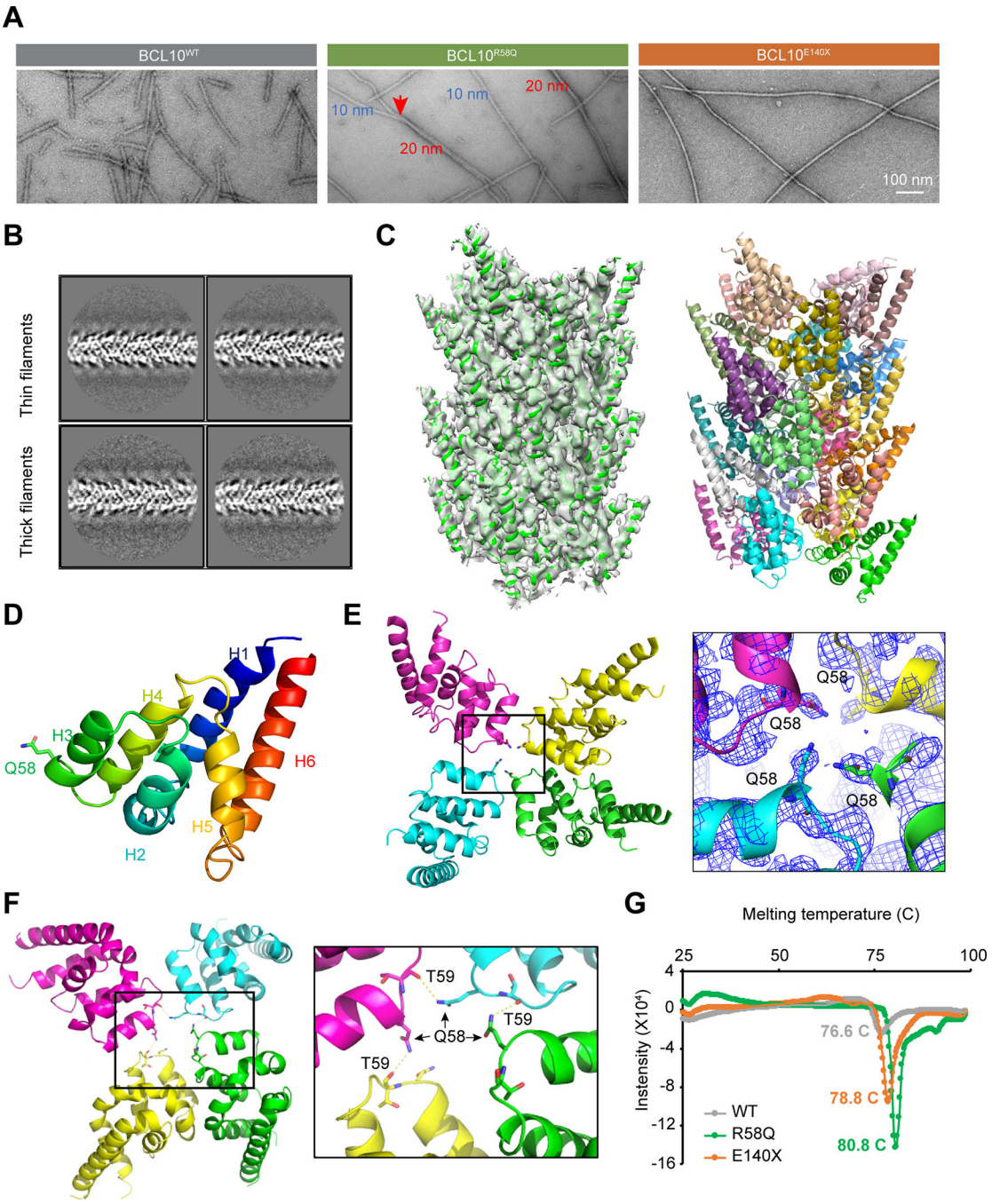

Figure 3 - CryoEM structure of BCL10^{R58Q} filamentation.

A. Negative stained EM micrographs of WT, R58Q and E140X filaments. R58Q formed a mixture of thin (10 nM) and thick (20 nM). Whereas E140X formed only thin filaments similar to WT. Scale bar 100 nM.

B. Representative 2D classes of BCL10 R58Q thin and thick filaments.

C. CryoEM structure of BCL10 R58Q at 4.6 Å. Left, BCL10 R58Q filament fitted into the CryoEM map. Right, BCL10 R58Q 16mer filament in which each subunit is colored differently.

D. BCL10 R58Q monomer. 58Q residue is labeled as stick.

E. BCL10 R58Q layer, showing 58Q residues facing each other for stabilizing Type III intrastrand Interface (left). Zoom in of BCL10 Q58 fitted into cryoEM density (right).

F. Potential hydrogen bonding network formed by the Q58 side chain of one protomer and the carbonyl oxygen of T59 from the next protomer in the helical spiral, shown on 4 consecutive R58Q subunits in the filament (left), and as a zoom-in view (right). These interactions stabilize the Type III intrastrand Interface.

G. Filament thermal stability assay performed by thermal shift assay for WT, E140X and R58Q purified filaments. E140X and R58Q filaments showed a significant shift in 2.1 °C and 4.2 °C, respectively in comparison to WT filaments.

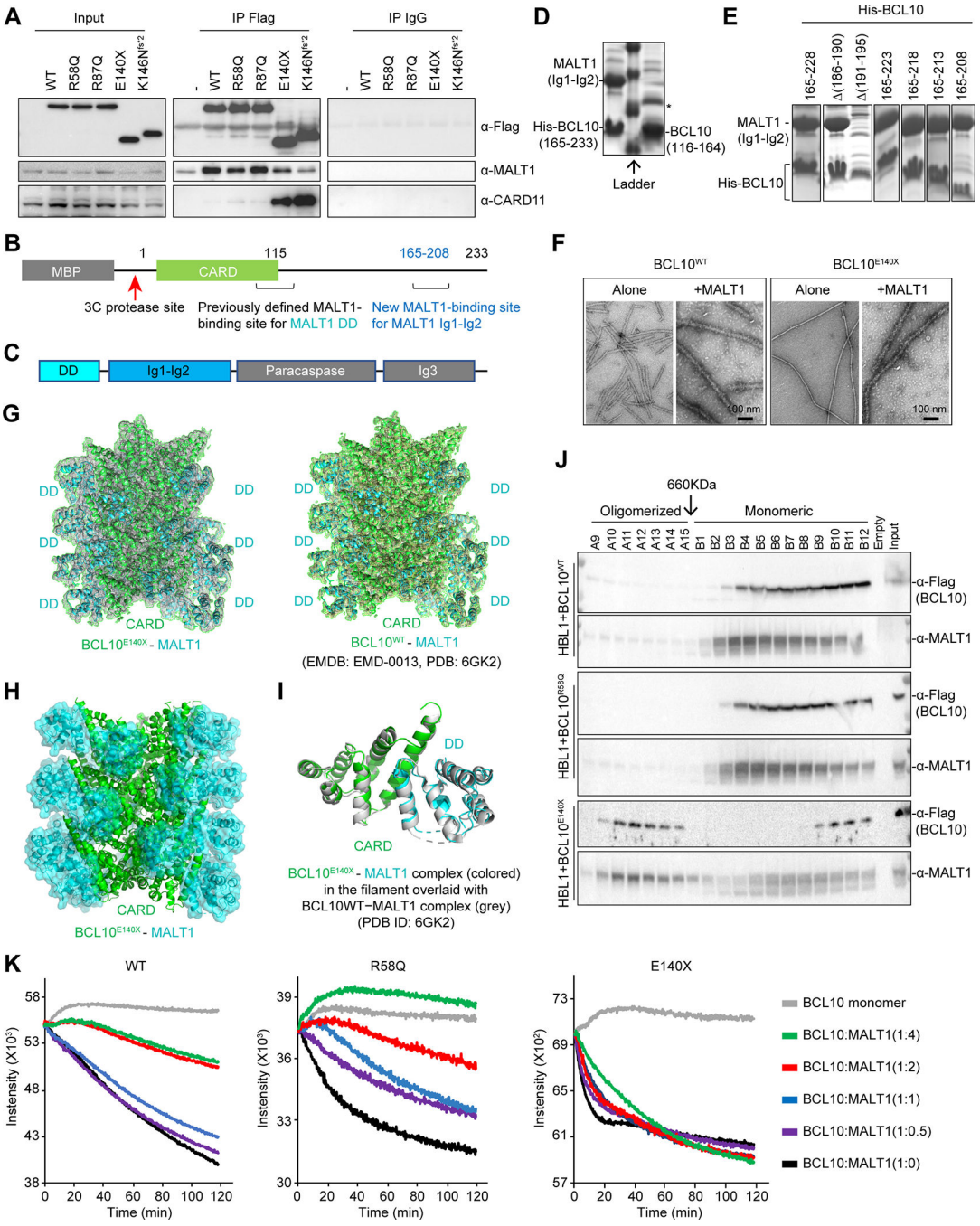

Figure 4 - CryoEM structure of BCL10^{E140X} filamentation.

A. Immunoblot analysis of BCL10 interactors performed by co-immunoprecipitating with anti-Flag antibody in Raji cells overexpressing either Flag-BCL10^{WT} or Flag-BCL10^{mutant} protein. Samples were blotted for anti-Flag, anti MALT1 and anti-CARD11. Input was loaded with 1% of total cell lysate used for IP. Anti-IgG antibody was used as negative control for CoIP.

B. Domain organization of MBP-human BCL10 construct mapped with MALT1 previously defined and new binding sites.

C. Domain organization of human MALT1 construct.

D. SDS-PAGE of MALT1 (Ig1-Ig2) pulldown by His-tagged BCL10 (165–233) (left) and His-tagged BCL10 (116–164) (right). * indicates a contaminant.

E. SDS-PAGE of MALT1 (Ig1-Ig2) pulldown by different truncations of His-tagged BCL10.

F. Negative stained EM micrographs of purified BCL10 WT filaments alone and with MALT1 (left) in comparison to BCL10 E140X filaments alone and with MALT1 filaments (right), resulted in similar filaments. Scale bar 100 nM.

G. CryoEM structure of BCL10 E140X - MALT1 DD filament at 4.3 Å fitted into the cryoEM density map (left). The 4.3 Å structure is similar to the previous published BCL10 WT CARD-MALT1 DD structure at 4.9 Å (right). However, BCL10 E140X -MALT1 DD shows improved density for MALT1 DD domain.

H. CryoEM structure of BCL10 E140X CARD and MALT1 DD (cyan) filament, emphasizing EM density for MALT1 DD.

I. Monomeric BCL10 E140X CARD-MALT1 DD (cyan) align to published monomeric BCL10 WT CARD - MALT1 DD.

J. Western blot for gel filtration fractions of HBL1 cells stably expressing Flag tagged BCL10 WT, E140X and R58Q. Fractions were blotted for anti-Flag and anti-MALT1. BCL10 E140X formed highly ordered oligomers co-migrated with MALT1, isolated from the void fractions.

K. MALT1 inhibits BCL10 filament formation through BCL10 C-terminal binding site. Quenching polymerization was measured for purified Alexa488 labeled BCL10 WT, E140X and R58Q at 3μM in the presence of increasing amounts of MALT1 (0μM, 1.5μM, 3μM, 6μM and 12μM). The assay was initiated upon addition of the 3C protease, in order to remove MBP tag from BCL10 WT, E140X and R58Q for allowing filament polymerization. Quenching was monitored for 2 h with 30 sec intervals by using Neo Biotek plate reader and performed with 3 biological replicates. Titration of increasing doses of MALT1 suppressed filament polymerization of fluorescently labeled BCL10 WT and R58Q. However, increasing doses of MALT1 had very little effect on E140X filament polymerization.

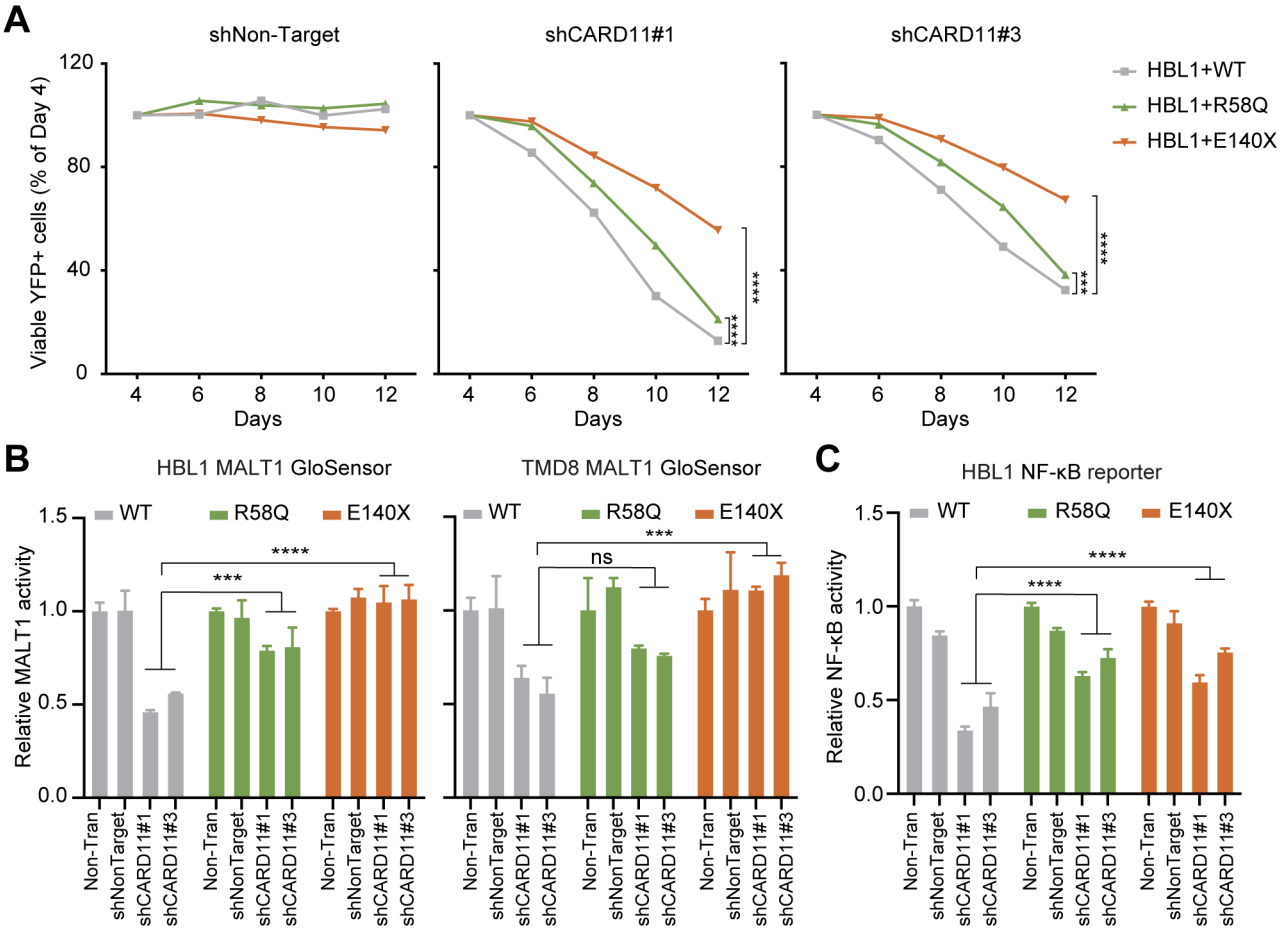

Figure 5 - BCL10 mutations are less dependent on upstream CARD11.

A. Viability of HBL1 lymphoma cell lines transduced to express shRNA targeting CARD11 with two independent hairpins or non-targeting control. The indicated lines stably expressing wildtype and mutant BCL10, were transduced with lentiviruses expressing *CARD11* shRNA along with YFP. The relative number of YFP+ live cells was plotted by normalizing them to Day 4 (the YFP+ peak). ***p<0.001; ****p<0.0001.

B. MALT1 activity using the MALT1 GloSensor reporter cells with CARD11 knockdown. The indicated MALT1 GloSensor cell lines were stably expressing wildtype and mutant BCL10, then transduced with lentiviruses expressing non-targeting or 2 independent *CARD11* hairpins coexpressing YFP reporter. At day 4, cells were harvested for MALT1 activity assay. Error bars indicate SEM with 4 biological replicates. ***p<0.001; ****p<0.0001; ns, not significant.

C. NF-κB activity in lymphoma reporter cells with shCARD11. The HBL1 NF-κB reporter cells were stably expressing wildtype and mutant BCL10, then transduced with lentiviruses expressing non-targeting or 2 independent *CARD11* hairpins coexpressing YFP reporter. NF-κB activity was measured 72 h posttransduction. Error bars indicate SEM with 4 biological replicates. ****p<0.0001.

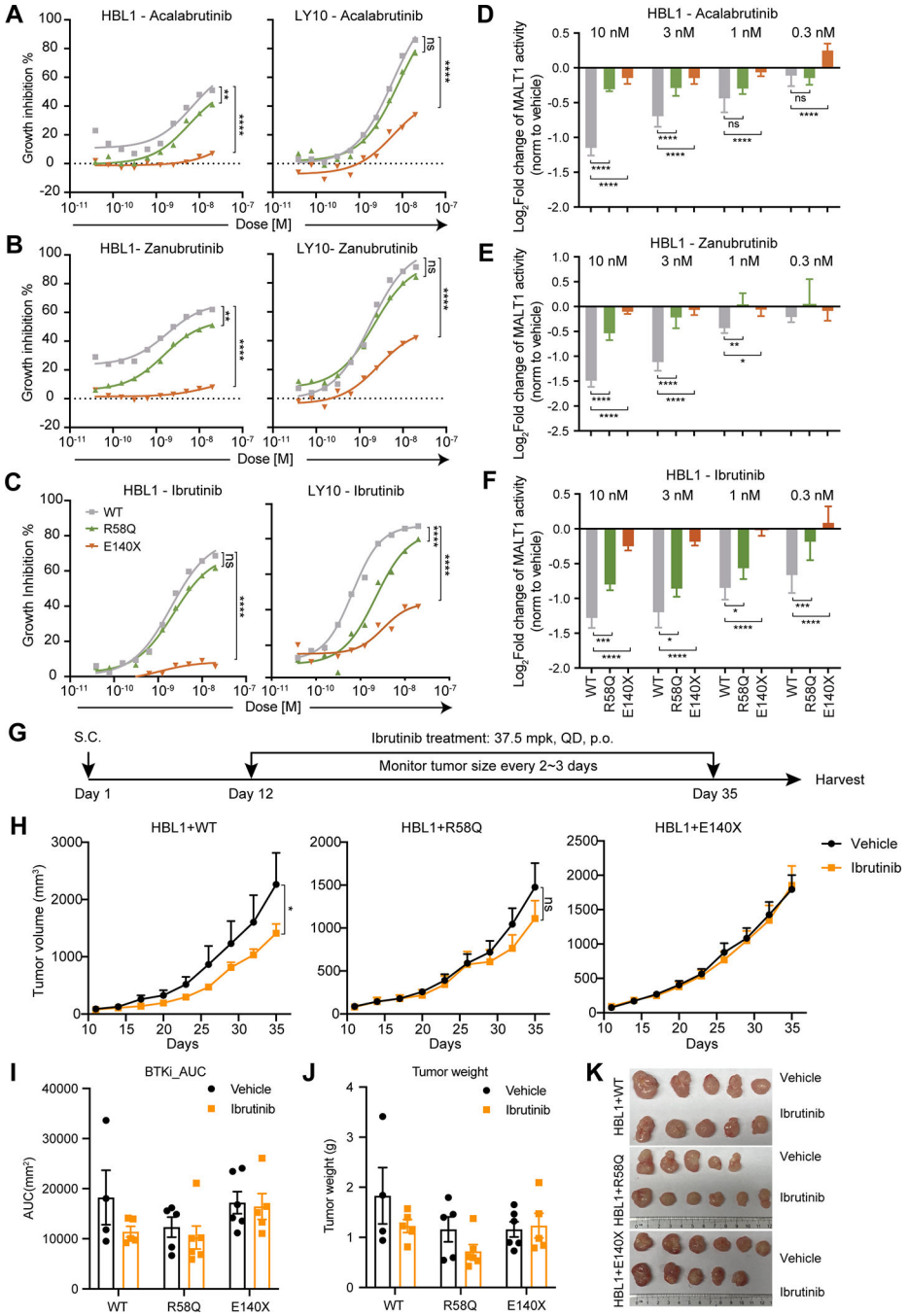

Figure 6 - BCL10 Gain-of-function mutant lymphomas are resistant to upstream BTK inhibitors.

A-C. Growth inhibition assay of lymphoma cells expressing WT or mutant BCL10 in response to BTK inhibitors. X axis, concentration of compound (M); Y axis, inhibition of cell growth normalized to vehicle treated cells. *p<0.05; **p<0.01; ****p<0.0001; ns, not significant.

D-F. Luciferase activity measured in the MALT1 GloSensor reporter cell lines with BTK inhibitor treatment. Indicated MALT1 GloSensor reporter lines were stably expressing

wildtype and mutant BCL10, and then treated with BTK inhibitors of different range (10 – 0.3 nM). NF-κB activity was performed 24 h after treatment. Error bars indicate SEM with 4 biological replicates. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001; ns, not significant.

G. Experimental design of xenografts with ibrutinib treatment.

H. Tumor growth curve for xenografts of HBL1 cells expressing WT and mutant BCL10 treated with vehicle and ibrutinib (n=5~6/group). Mice were treated orally with 25 mg/kg ibrutinib once per day for 24 consecutive days.

I. Analysis of area under the curve (AUC) in **h**. AUC is calculated with Prism.

J. Tumor weight measured at the endpoint.

K. Representative photos of tumors harvested at endpoint.

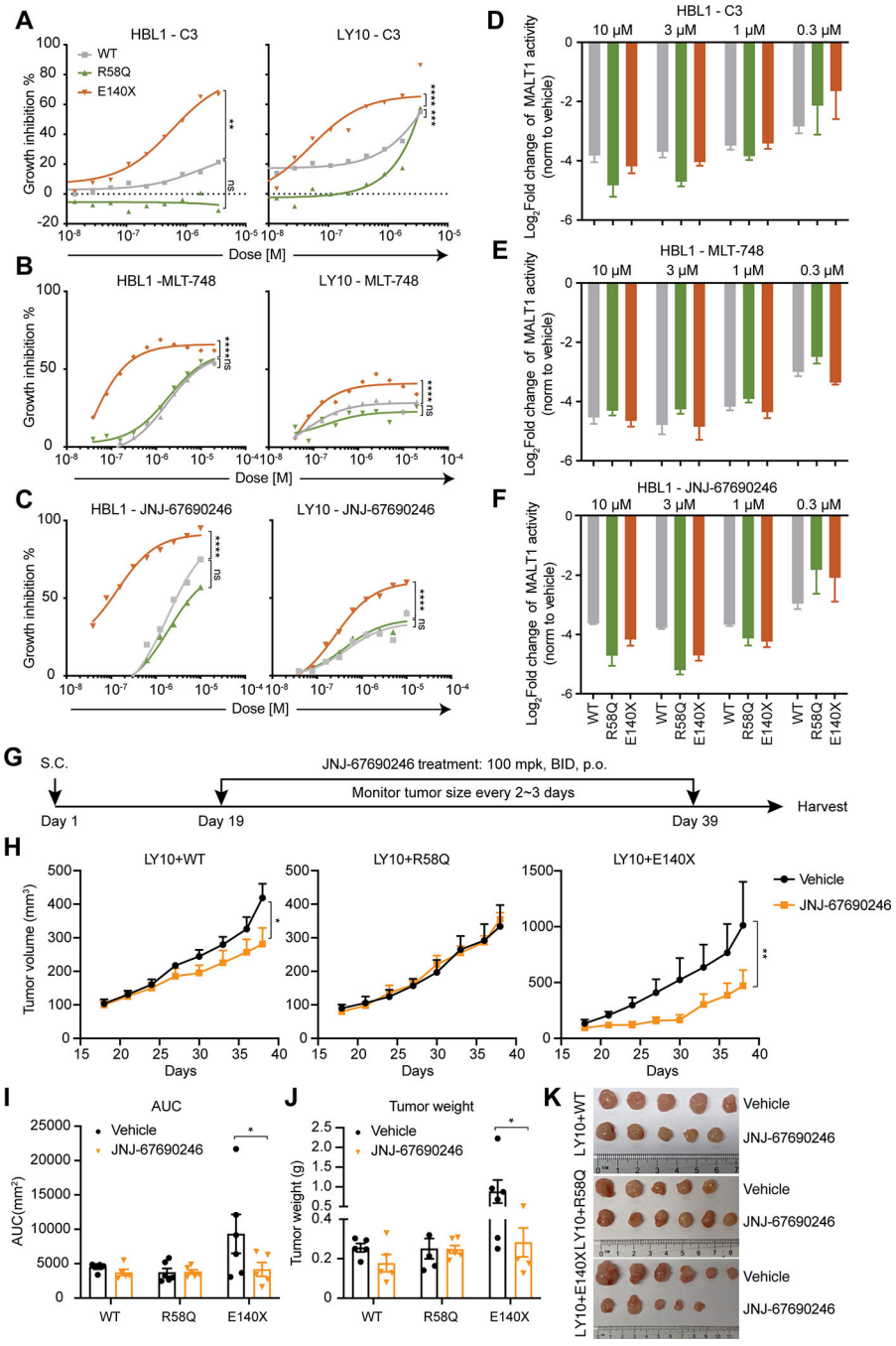

Figure 7 - BCL10 truncating mutant lymphomas are hypersensitive to MALT1 protease inhibitors.

A-C. Growth inhibition assay of lymphoma cells expressing WT or mutant BCL10 in response to MALT1 inhibitors. X axis, concentration of compound (M); Y axis, inhibition of cell growth normalized to vehicle treated cells. **p<0.01; ***p<0.001; ****p<0.0001; ns, not significant.

D-F. Luciferase activity measured in the MALT1 GloSensor reporter cell lines with MALT1 inhibitor treatment. Indicated MALT1 GloSensor reporter lines were stably expressing

wildtype and mutant BCL10, and then treated with MALT1 inhibitors of different range (10 – 0.3 uM). NF-κB activity was performed 24 h after treatment. Error bars indicate SEM with 4 biological replicates.

G. Experimental design of xenografts with JNJ-67690246 treatment.

H. Tumor growth curve for xenografts of HBL1 cells expressing WT and mutant BCL10 treated with vehicle and JNJ-67690246 (n=3~6/group). Mice were treated orally with 100 mg/kg twice per day for 19 consecutive days.

I. Analysis of area under the curve (AUC) in **h**. AUC is calculated with Prism. *p<0.05.

J. Tumor weight measured at the endpoint. *p<0.05.

K. Representative photos of tumors harvested at endpoint.