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Comprehensive genomic profiling of orbital and ocular adnexal lymphomas identifies frequent alterations in *MYD88* and chromatin modifiers: new routes to targeted therapies

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Non-Hodgkin lymphoma of the orbit and ocular adnexa is the most common primary orbital malignancy. Treatments for low- (extra-nodal marginal zone and follicular lymphomas) and high-grade (diffuse large B-cell lymphoma) are associated with local and vision-threatening toxicities. High-grade lymphomas relapse frequently and exhibit poor survival rates. Despite advances in genomic profiling and precision medicine, orbital and ocular adnexal lymphomas remain poorly characterized molecularly. We performed targeted next-generation sequencing (NGS) profiling of 38 formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas obtained from a single-center using a panel targeting near-term, clinically relevant genes. Potentially actionable mutations and copy number alterations were prioritized based on gain- and loss-of-function analyses, and catalogued, approved, and investigational therapies. Of 36 informative samples, including marginal zone lymphomas ($n=20$), follicular lymphomas ($n=9$), and diffuse large B-cell lymphomas ($n=7$), 53% harbored a prioritized alteration (median = 1, range 0–5/sample). *MYD88* was the most frequently altered gene in our cohort, with potentially clinically relevant hotspot gain-of-function mutations identified in 71% of diffuse large B-cell lymphomas and 25% of marginal zone lymphomas. Prioritized alterations in epigenetic modulators were common and included gain-of-function *EZH2* and loss-of-function *ARID1A* mutations (14% of diffuse large B-cell lymphomas and 22% of follicular lymphomas contained alterations in each of these two genes). Single prioritized alterations were also identified in the histone methyltransferases *KMT2B* (follicular lymphoma) and *KMT3B* (diffuse large B-cell lymphoma). Loss-of-function mutations and copy number alterations in the tumor suppressors *TP53* (diffuse large B-cell and follicular lymphoma), *CDKN2A* (diffuse large B-cell and marginal zone lymphoma), *PTEN* (diffuse large B-cell lymphoma), *ATM* (diffuse large B-cell lymphoma), and *NF1* (diffuse large B-cell lymphoma), and gain-of-function mutations in the oncogenes *HRAS* (follicular lymphoma) and *NRAS* (diffuse large B-cell lymphoma) were also observed. Together, our study demonstrates that NGS can be used to profile routine formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas for identification of somatic-driving alterations and nomination of potential therapeutic strategies.
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Introduction

Non-Hodgkin lymphomas (NHLs) of the orbital and ocular adnexa represent 10–15% of all tumors present in the orbit, eyelids, conjunctiva, and lacrimal apparatus (ie, the 'adnexa') and are the most common primary orbital cancer in adults.[1,2] Alarmingly, orbital and ocular adnexal lymphomas have shown an increasing incidence in some populations (~6% yearly increase in white Americans from an analysis of Surveillance, Epidemiology, and End Results data).[3]

Orbital and ocular adnexal lymphomas are B-cell proliferations that include low-grade tumors such as extra-nodal marginal zone (also known as mucosa-associated lymphoid tissue lymphoma (MALT)) and follicular lymphoma, as well as high-grade tumors such as mantle cell lymphoma and diffuse large B-cell lymphomas. These subtypes account for ~50%, 10–20%, 5–10%, and 5–10% of orbital and ocular adnexal lymphomas, respectively.[4,5]

The molecular etiology of orbital and ocular adnexal lymphoma heterogeneous subtypes is diverse and not well understood. MZLs are frequently associated with translocations: *API2-MALT1*, *IGH-MALT1*, *IGH-BCL10*, and *IGH-FOXP1*, as well as trisomy of chromosome 3 and 18.[6] Such aberrations activate pathways that converge on the nuclear factor-κB (NF-κB) complex, leading to orbital and ocular adnexal lymphomagenesis.[6] Molecular studies of orbital and ocular adnexal follicular and diffuse large B-cell lymphomas are few, owing to the rarity of primary tumors of these subtypes in these locations. Thus, molecular alterations in these subtypes are presumed to follow systemic FL and DLBCL pathogenetic patterns. In systemic diffuse large B-cell lymphomas and *BCL2/IGH* and *MYC* rearrangements, lesions associated with NF-κB activation (*TNFAIP3*, *CARD11*, *CD79B*, and *MYD88*) and epigenetic alterations (*CREBBP*, *EP300*, and *EZH2*) have been described, but there are also many altered genes that encode for structural (eg, *PCLO*) and signaling proteins (*P2RY8* and *TNFRSF14*).[7,8] Systemic follicular lymphomas share some lesions with diffuse large B-cell lymphomas, including *IGH* and *BCL2* rearrangements[9,10] and alterations in *EZH2* and *TNFRSF14*,[11] but also exhibits distinct changes including somatic mutations that lead to loss of function of *EPHA2* ephrin receptor[12] and acquisition of *N*-glycosylation motifs in immunoglobulin genes.[13]

Although the mainstay of treatment for most primary orbital and ocular adnexal lymphomas (low grade), external beam radiation therapy, results in high rates of local control, the rate of distant relapse can reach 40%[14,15] and local toxicities such as keratitis, dry eye, cataract, neovascular glaucoma, optic nerve neuropathy, and retinopathy can lead to vision loss or blindness.[16,17] Survival rates vary with orbital and ocular adnexal lymphoma subtype. In general, marginal zone and most follicular lymphomas (low-grade tumors) of the orbital and ocular adnexal region are treated with external beam radiation therapy and 10-year disease-specific survival rates, depending on stage, range from 94 to 98%.[18,19] In contrast, diffuse large B-cell lymphomas and some follicular lymphomas of the orbital and ocular adnexal region are tumors that often lead to, or are a manifestation of, disseminated systemic disease and are treated with a combination of external beam radiation therapy, surgery, and/or systemic chemotherapy. These diffuse large B-cell lymphomas relapse frequently and have 10-year disease-specific survival rates ranging from 18 to 55%.[20]

In summary, the genomic drivers of orbital and ocular adnexal lymphomas, especially those that underlie orbital and ocular adnexal follicular and diffuse large B-cell lymphomagenesis remain poorly understood, due, in part, to the rarity of primary tumors at these locations. Although low-grade tumors such as marginal zone and follicular lymphomas are frequently curable by external beam radiation therapy, they are often associated with vision-threatening local toxicities and can relapse at distant locations. Treatments such as external beam radiation therapy, surgery and systemic chemotherapy for high-grade orbital and ocular adnexal lymphomas are associated with vision threatening and systemic toxicities. Moreover, diffuse large B-cell lymphomas frequently relapse and are often deadly. Taken together, these data demonstrate an unmet need to develop novel treatment strategies for both low- and high-grade orbital and ocular adnexal lymphomas.

The development of novel therapies for orbital and ocular adnexal lymphomas has been hampered by the limited nature of studies exploring genomic drivers of the disease. Although cytogenetic, copy number and transcript, and immunohistochemistry-based profiling studies have been reported,[21–25] these represent limited cohorts mainly assessing marginal zone lymphoma, the orbital and ocular adnexal lymphoma subtype most frequently associated with good prognosis. Importantly, although next-generation sequencing (NGS) studies have defined genetic alterations and potential therapeutic targets in non-orbital lymphomas, such approaches have not been applied to orbital and ocular adnexal lymphomas. Hence, in order to define potentially actionable somatic mutations and copy number alterations in orbital and ocular adnexal lymphomas, we performed NGS on 38 formalin-fixed, paraffin embedded orbital and ocular adnexal lymphomas, including marginal zone, follicular and diffuse large B-cell histologic subtypes. To do this, we subjected our set of orbital and ocular adnexal lymphomas to a NGS-based panel enriched for alterations in solid tumors and lymphomas, a modified version of which is being used in the National Cancer Institute-Molecular Analysis for Therapy Choice (NCI-MATCH) Trial,[26] a new trial that has matched

patients with advanced lymphomas and solid tumors, which have not responded (or never responded), to approved or investigational therapeutics based on their prioritized mutation profile rather than the site of tumor origin.[27]

Materials and methods

Case Selection

The study was carried out at the highest ethical standards and with the approval of the University of Michigan Institutional Review Board. We identified a cohort of 38 archived, routine clinical formalin-fixed, paraffin-embedded orbital lymphoma specimens from the University of Michigan Department of Pathology Tissue Archive for NGS. Clinicopathological information for each case was obtained from the clinical archive. Hematoxylin and eosin (H&E)-stained slides and immunostains (where available) were reviewed for all cases by board-certified Anatomic Pathologists (ASM and SAT), to ensure sufficient tumor content (70–90%).

Targeted NGS

Targeted NGS was performed essentially as previously described.[26,28,29] For each specimen, $3–7 \times 10\,\mu m$ formalin-fixed, paraffin-embedded sections were cut from a single representative block per case, using macrodissection with a scalpel as needed, to enrich for tumor content. DNA was isolated using the Qiagen Allprep formalin-fixed, paraffin-embedded DNA/RNA kit (Qiagen, Valencia, CA) and quantified as previously described. Targeted, multiplexed PCR-based NGS was performed by Ion Torrent NGS using the DNA component of a beta version of the Oncomine Comprehensive Assay, a custom panel comprising 3435 amplicons targeting 126 genes. Genes included in this panel were selected based on pan-solid tumor NGS and copy number profiling data analysis that prioritized somatic, recurrently altered oncogenes, tumors suppressors, genes present in high-level copy gains/losses, and known/investigational therapeutic targets.[26] Library preparation with barcode incorporation, template preparation, and sequencing using the Ion Torrent Proton sequencer were performed according to the manufacturer's instructions. Data analysis was performed using Torrent Suite 4.0.2, with alignment by TMAP using default parameters, and variant calling using the Torrent Variant Caller plugin (version 4.0-r76860) using default low-stringency somatic variant settings. Variant annotation filtering and prioritization was performed essentially as described, using validated in-house pipelines.[26,28–31] Briefly, called variants were filtered to remove synonymous or non-coding variants, those with flow-corrected read depths (FDP) < 20, flow-corrected variant allele containing reads (FAO) < 6, variant allele frequencies (FAO/FDP) < 0.10, extreme skewing of forward/reverse flow-corrected reads calling the variant (< 0.2 or > 5), or indels within homopolymer runs > 4. Called variants were filtered using a panel-specific, in-house blacklist. Variants with allele frequencies $> 0.5\%$ in Exome Sequencing Project 6500 (ESP6500) or the 1000 Genomes project, and those reported in ESP6500 or 1000 genomes with observed variant fractions between 0.40 and 0.60 or > 0.9 were considered germline variants unless occurring at a known hotspot variant. Variants located at the last mapped base (or outside) of amplicon target regions, variants with the majority of supporting reads harboring additional mismatches or indels (likely sequencing error), those in repeat-rich regions (likely mapping artifacts), and variants occurring exclusively in one amplicon if overlapping amplicons cover the variant were excluded. High-confidence somatic variants passing the above criteria were then visually confirmed in Integrative Genomics Viewer (https://www.broadinstitute.org/igv/). We have previously confirmed that these filtering criteria identify prioritized high-confidence somatic variants that pass Sanger sequencing validation with $> 95\%$ accuracy.[28–32] Copy number analysis from total amplicon read counts provided by the Coverage Analysis Plug-in (v4.0-r77897) was performed essentially as described using a validated approach.[26,28,29,33] Genes with a $\log_2$ copy number estimate of < -1 or > 0.6 were considered to have high-level loss or gain, respectively.

To prioritize potential driving alterations, we used Oncomine software tools (powertools.oncomine.com) to annotate called variants, which use pan-cancer NGS data to identify genes as oncogenes or tumor suppressors, based on overrepresentation of hotspot or deleterious mutations, respectively. Variants in oncogenes are then considered gain-of-function if at a hotspot and variants in tumor suppressors are considered loss-of-function if deleterious or at a hotspot.[26,28,30] Likewise, high-level copy number alterations were prioritized if they were concordant with the minimal common region analysis used to design the Oncomine Comprehensive Assay (eg, high-level copy number gain in a gene prioritized as amplified/deleted by minimal common region analysis.

Immunohistochemistry

Immunohistochemistry was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO Envision+ and diaminobenzadine as the chromogen. Sections of de-paraffinized Orbital and ocular adnexal lymphomas were labeled with ARID1A (mouse monoclonal, clone PSG3, 1:250, Santa Cruz Biotechnology, Dallas TX, sc-32761), EZH2 (mouse monoclonal, clone 11, 1:100, BD Biosciences, San Jose, CA, 612666), or KMT3B/NSD1 (rabbit polyclonal, 1:250, EMD Millipore, Billerica, MA,

ABE1009) for 60 min at ambient temperature. Microwave epitope retrieval in 10 mM Tris/HCl pH 9 containing 1 mM EDTA was used before staining. Appropriate negative (no primary antibody) and positive controls were stained in parallel with each set of tumors studied. Immunostained slides were examined by light microscopy by two pathologists (A.S.M. and S.A.T.). Only staining of the nucleus was marked as a positive expression. The staining was scored semi-quantitatively and recorded based on percent nuclei staining (0 = negative, 1 = 1–25% immunoreactive cells, 2 = 26–50% immunoreactive cells, 3 = 51–75% immunoreactive cells, and 4 = 76–100% immunoreactive cells) and intensity of staining (0 = negative, 1 = weak, 2 = moderate, and 3 = strong). Corresponding sections were also H&E stained.

Statistics

Comparisons of the number of mutations and copy number alterations per sample by lymphoma subtype were performed using the Kruskal–Wallis test with *post-hoc* pairwise comparison of subgroups using R 3.1.0 (R Foundation for Statistical Computing, Vienna, Austria).

Results

We performed targeted NGS on a cohort of 38 formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas comprising 8 diffuse large B-cell, 9 follicular, and 21 marginal zone lymphomas; representative photomicrographs are shown in Figure 1a and b and clinical characteristics of all informative patients (see below) are presented in Figure 1c. We isolated an average of 5.5 μg DNA per case (range 0.1–24.1 μg) and all samples had $>70\%$ estimated tumor content by H&E evaluation after macrodissection (as needed). NGS was performed using the DNA component of a beta version of the Oncomine Comprehensive Assay (version 1), a custom panel comprising 3435 amplicons targeting 126 genes, and Ion Torrent-based sequencing on the Proton machine, a modified version of which is being used in the NCI-MATCH trial.[27] Targeted genes were selected based on pan-solid tumor NGS and copy number profiling data analysis, to prioritize somatic, recurrently altered oncogenes, tumors suppressors, and genes present in high-level copy number alteration, filtered by available or investigational therapeutic targets.[26]

Of the 38 samples assayed, one marginal zone lymphoma (IE-26, primary to orbit/ocular adnexa, Ann Arbor stage IIAE, with no previous treatment) and one diffuse large B-cell lymphoma (IE-31, non-primary to orbit/ocular adnexa, Ann Arbor stage IAE, with previous chemotherapy and EBRT) sample yielded low-quality sequencing data due to poor genomic DNA quality and were excluded from all subsequent analyses (Figure 1c). Most of the informative 36 lymphomas were primary to the orbit or ocular adnexa, not previously treated, and of early stage. Twenty-seven of 36 tumors were primary to the orbit or ocular adnexa, including 6 of 7 diffuse large B-cell lymphomas, 4 of 9 follicular lymphomas, and 17 of 20 marginal zone lymphomas. Most were not previously treated: none of the diffuse large B-cell lymphomas, 5 of the follicular lymphomas, and 18 of the marginal zone lymphomas. Most of the marginal zone lymphomas[15] and diffuse large B-cell lymphomas[6] were Ann Arbor stage IAE, whereas most of the follicular lymphomas were IAE[2] or IIAE[5] (Figure 1c). Primary follicular and diffuse large B-cell lymphomas to the orbit and ocular adnexa have rarely been profiled.

Using the Hans algorithm for germinal center B-cell phenotyping, we found that six of the eight diffuse large B-cell lymphomas were of the activated B-cell like phenotype and the remaining two were of the germinal center B-cell phenotype.[34] One of the germinal center B-cell-positive cases was unable to be sequenced due to poor DNA quality (IE-31).

Across the 36 informative samples, NGS generated an average of 1 871 816 mapped reads yielding 545 × targeted base coverage (Supplementary Table S1). We identified a total of 41 high-confidence, prioritized somatic alterations (median $n=1$, range $n=0$–5 per sample) comprising nonsynonymous point mutations ($n=33$), short insertions/deletions (indels; $n=2$), and copy number variations ($n=6$). All clinical data (including staging, primary site, and previous treatment), prioritized somatic mutations, and high-level copy number alterations for each case are shown in an integrative heat map (Figure 1c) and given in Supplementary Table S2. Prioritized alterations were present in 19 of 36 samples (53%). Diffuse large B-cell lymphomas harbored the majority of total prioritized alterations (6 of 7 (86%) with prioritized alterations; median prioritized alterations $n=3$, range $n=0$–5). Prioritized alterations were identified in 6 of 9 (67%) follicular lymphomas (median prioritized alterations $n=1$, range $n=0$–3) and 7 of 20 (35%) marginal zone lymphomas (median prioritized alterations $n=0$, range $n=0$–2). The number of prioritized alterations was significantly different between the lymphoma subtypes (Kruskal–Wallis test, $P=0.0014$), as shown in Figure 1d.

Across the 36 orbital and ocular adnexal lymphomas, 10 (28%) harbored prioritized gain-of-function mutations in the Toll/interleukin-1 receptor (TIR) domain of myeloid differentiation factor 88 (*MYD88*), making it the most frequently altered gene (by prioritized alterations) in our cohort. MYD88 is an adaptor protein that binds to the intracellular domains of Toll-like receptors (TLRs) and interleukin 1 receptor on B cells and macrophages, which stimulates the NF-κB signaling pathway, and is involved in innate immunity.[35] The highest proportion of *MYD88* hotspot gain-of-function mutations was present in diffuse large B-cell lymphomas

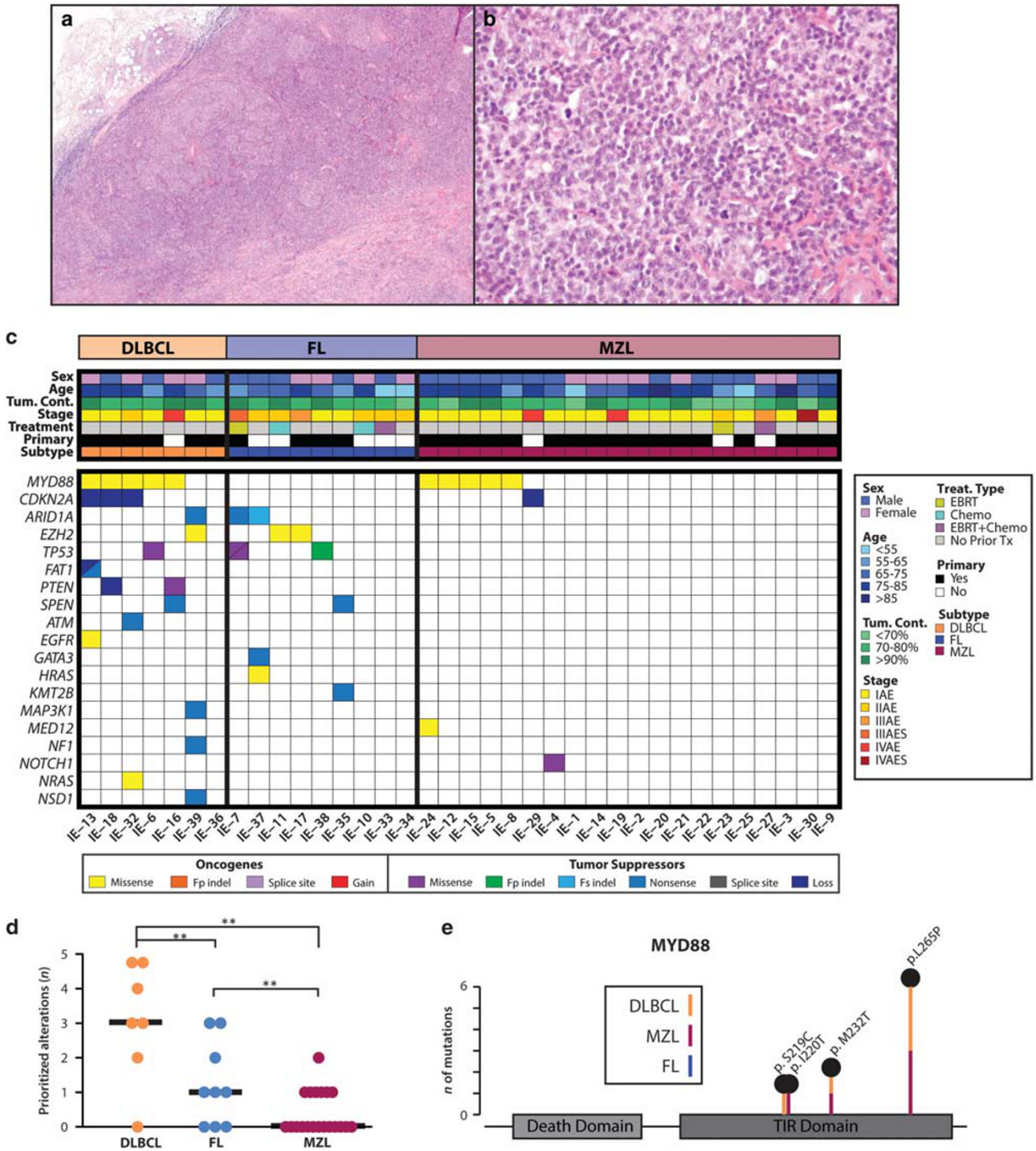


Figure 1 Targeted next-generation sequencing (NGS) of routine formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas identifies recurrent informative/potentially actionable alterations. (**a** and **b**) Representative histology by hematoxylin and eosin (H&E) staining of a formalin-fixed, paraffin-embedded lymphoma sample (IE-38, a follicular lymphoma (FL)). Original magnification, ×4 (**a**) and ×40 (**b**). (**c**) Integrative heatmap of prioritized, informative/potentially actionable mutations and copy number alterations across the 36 informative orbital and ocular adnexal lymphomas. Clinicopathologic information including patient sex, age, estimated tumor content, and subtypes (diffuse large B-cell lymphoma (DLBCL), FL, and marginal zone lymphoma (MZL)) are indicated in the header according to the legend on the right. All prioritized, high-confidence, gain-of-function or loss-of-function somatic mutations in oncogenes and tumor suppressors, respectively, as well as high-level copy number alterations are shown for each case. Specific alteration types are indicated according to the legend (Fp and Fs indel, frame-preserving and frame-shift indels, respectively; Gain, copy number gain; Loss, copy number loss). Slashed boxes indicate two alterations. (**d**) Comparison of the number (*n*) of prioritized alterations per sample (mutations and copy number alterations) by subtype. The median number of alterations per type is shown by thick black bars. $^{**}P < 0.01$ from *post-hoc* subgroup comparison of Kruskal–Wallis test. (**e**) Stick plot of recurrent domain mutations. Schematic representation of the location and subtype of *MYD88* gain-of-function mutations identified in 10 of 36 orbital and ocular adnexal lymphomas. The number of mutations (*n*) at each residue is indicated by the stick length according to the scale (left).

(5/7, 71%), including three p.L265P, one p.M232T and one p.S219C mutations. Notably, of the five *MYD88* mutation-positive diffuse large B-cell lymphomas, all were of the activated B-cell-like phenotype. The former represents the most frequently altered *MYD88* hotspot across human cancers, whereas the latter two mutations occurred at minor hotspots. The remaining *MYD88* (TIR domain) mutations occurred in 5 of 20 (25%) marginal zone lymphoma samples. Of note, *MYD88* mutations comprise the majority of total prioritized alterations (5 of 8, 63%) in our marginal zone lymphoma samples, with three p.L265P mutations and two other TIR domain hotspot mutations (one each of p. M232T and p.I220T). All gain-of-function *MYD88* mutations across the cohort are shown in Figure 1e. Taken together, our data demonstrate that *MYD88* TIR domain mutations are common in the diffuse large B-cell and marginal zone lymphoma subtypes of orbital and ocular adnexal lymphomas.

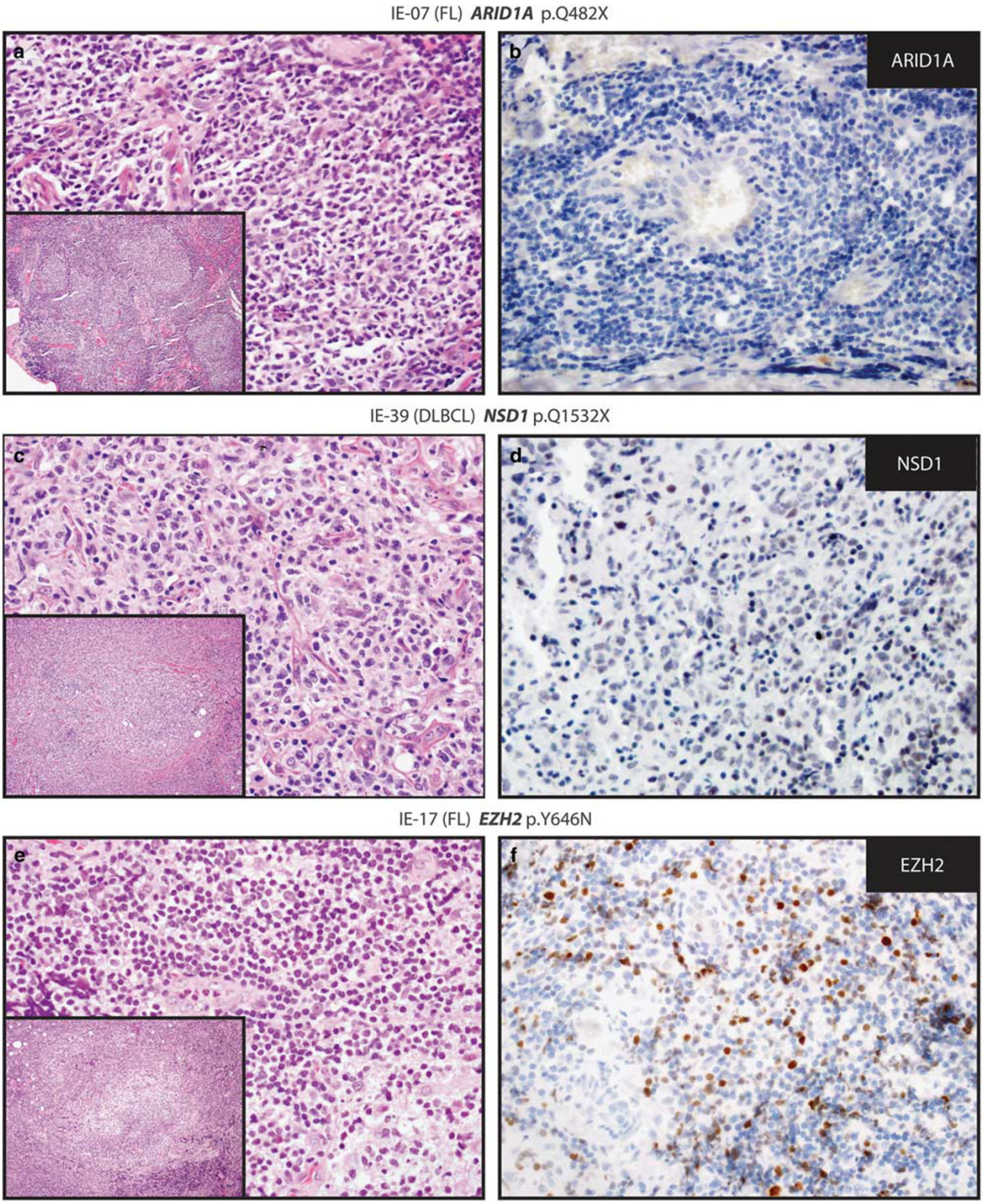
Recurrent somatic alterations in histone/chromatin remodeling proteins are frequent in extranodal NHLs outside of the orbit and ocular adnexa. Specifically, 22% of diffuse large B-cell lymphomas and 7–22% of follicular lymphomas outside of the orbit and ocular adnexa carry gain-of-function missense mutations (at the p.Y646 hotspot) in the methyltransferase SET domain of the histone H3 lysine 27 trimethylase enhancer of zeste homolog 2 (*EZH2*).[36–38] Herein, we identified two of nine follicular lymphomas (22%) and one of seven diffuse large B-cell lymphomas (14%) with *EZH2* p.Y646 mutations (Figure 1c). The single *EZH2* mutant diffuse large B-cell lymphoma case in our cohort was of the germinal center B-cell phenotype. We also identified loss-of-function mutations in tumor suppressor AT-rich interactive domain-containing protein 1A (*ARID1A)*, an epigenetic regulator that is an ATPase-dependent SWI/SNF nucleosome remodeling complex subunit. We identified a prioritized *ARID1A* nonsense mutation (p.Q1363X) in one of seven (14%) diffuse large B-cell lymphomas; two of nine (22%) follicular lymphomas harbored loss-of-function ARID1A mutations (p.Q482X and p.727_730del). Interestingly, the *ARID1A*-mutated diffuse large B-cell lymphoma sample (IE-39; p.Q1363X) also contained one of the aforementioned activating *EZH2* Y646 mutations (p.Y646S) (Figure 1c). Lastly, we identified prioritized mutations in other chromatin-modifying genes including loss-of-function mutations in *KMT2B* (also known as *MLL2* or *MLL4*; p.Q2495X) and *KMT3B* (also known as *NSD1*; p.Q1532X) in one case each of the follicular lymphoma and diffuse large B-cell lymphoma, respectively (Figure 1c).

All mutations in the above chromatin modeling genes in our cohort were heterozygous based on estimated tumor content and variant allele frequencies. Hence, to determine the impact of mutations on protein expression, we assessed ARID1A, EZH2 and KMT3B expression in several lymphoma samples by immunohistochemistry. Although limited by the number of mutant samples, immunohistochemical expression of ARID1A in wild-type and mutant follicular lymphoma samples showed no qualitative differences, as ARID1A protein was very low or absent in both samples (Figure 2a, positive control not shown). In contrast, the diffuse large B-cell lymphoma harboring the loss-of-function *KMT3B (NSD1)* p.Q1532X nonsense mutation showed decreased KMT3B expression compared with a diffuse large B-cell lymphoma sample without prioritized *KMT3B* alteration (Figure 2b). EZH2 expression was retained in two samples that each harbored prioritized p.Y646N or p.Y646S mutations, which are known gain-of-function mutations (Figure 2c).

In addition to *MYD88* and chromatin-modifying genes, we also identified samples with prioritized alterations in well-known tumor suppressors and oncogenes (Figure 1c and Supplementary Table S2). Three diffuse large B-cell lymphomas harbored single loss-of-function mutations in phosphatase and tensin homolog (*PTEN*), ataxia telangiectasia mutation (*ATM*), or neurofibromin 1 (*NF1*). Of interest, sample IE-07, a follicular lymphoma, harbored biallelic tumor protein 53 (*TP53*) loss-of-function hotspot mutations (R248 and G244). Another follicular lymphoma contained a *TP53* non-frameshift deletion (p.255-256del), while a diffuse large B-cell lymphoma harbored a *TP53* R175 hotspot missense mutation. Loss-of-function nonsense mutations were also observed in the tumor suppressor split end family protein (*SPEN*) in one case each of diffuse large B-cell lymphoma and follicular lymphoma. Prioritized alterations in oncogenes included missense mutations in neuroblastoma rat sarcoma viral oncogene homolog (*NRAS,* Q61K) and Harvey rat sarcoma viral oncogene homolog (*HRAS*, G60S) in a single diffuse large B-cell lymphoma and follicular lymphoma sample, respectively.

Figure 2 Immunohistochemistry for chromatin-modifying proteins in mutant *ARID1A*, *NSD1*, and *EZH2* lymphoma samples. Hematoxylin and eosin (H&E, **a**, **c** and **e**) stains (large panels, original magnifications: ×400, insets ×100) and corresponding immunohistochemistry (**b**, **d** and **f**, original magnifications: ×400) for indicated chromatin-modifying proteins was performed in selected orbital and ocular adnexal lymphomas based on mutational status. (**a** and **b**) IE-07, a usual appearing follicular lymphoma (**a**) with a loss-of-function *ARID1A* mutation (p.Q842X), demonstrates absent ARID1A expression (**b**). (**c** and **d**). IE-39, a diffuse large B-cell and marginal zone lymphoma with a truncating stopgain mutation in *NSD1* (p.Q1532X), shows very weak expression of NSD1 (**d**) in tumor cells (wild-type NSD1 diffuse large B-cell and marginal zone lymphoma samples showed robust expression of NSD1 by immunohistochemistry, see Supplementary Figure). IE-17, a follicular lymphoma (**e**) with a predicted gain-of-function variant in *EZH2* (p.Y646N), shows robust EZH2 (**f**) expression (wild-type *EZH2* marginal zone lymphoma samples did not show EZH2 expression by immunohistochemistry, see Supplementary Figure).

In addition to single nucleotide alterations and short indels, we used NGS to assess copy number alterations in genes targeted by our panel using a validated approach.[33] Overall, we identified relatively few focal, high-level amplifications or deletions in our orbital and ocular adnexal lymphoma cohort (Figure 1c). We identified high level, prioritized deletions in *CDKN2A* (*p16INK4A*) in three diffuse large B-cell lymphomas and one marginal zone lymphoma, as well as a high-level deletion in *PTEN*



in a diffuse large B-cell lymphoma. Finally, although not prioritized, broad low-level gains/losses were observed, including one-copy gains in chromosomes 12 and 3 in a diffuse large B-cell lymphoma and a marginal zone lymphoma, respectively, as well as one-copy losses in 17p in two diffuse large B-cell lymphomas. Cohort-wide copy number plots are shown in Figure 3a, with individual amplicon level plots for one sample each for diffuse large B-cell lymphoma, follicular lymphoma, and marginal zone lymphoma (Figure 3b). Among other copy number changes, we found high-level deletions in *CDKN2A* and *PTEN* (IE-18, diffuse large B-cell lymphoma), and CDKN2A (IE-29, marginal zone lymphoma), whereas no copy number gains or losses were observed in any of the follicular lymphomas profiled (IE-17, representative plot) (Figure 3b).

Discussion

We performed targeted NGS of 38 formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas (marginal zone, follicular, and diffuse large B-cell lymphoma subtypes), to identify somatic mutations and copy number alterations associated with tumorigenesis, and identify novel potential therapeutic strategies (Figure 4). Importantly, although numerous studies have profiled extraorbital and ocular adnexal NHLs and B-cell neoplasms using NGS, orbital and ocular adnexal lymphomas have not been profiled by comprehensive approaches, and it is unclear whether these cancers share similar alterations and potential therapeutic targets.[38–43]

The most frequently mutated gene in our cohort was *MYD88*, with 28% of orbital and ocular adnexal lymphomas harboring gain-of-function hotspot mutations. A major finding of this work was that the frequencies of *MYD88* mutations in marginal zone and diffuse large B-cell lymphomas of the orbital and ocular adnexal regions was higher than those previously reported for these subtypes in locations outside the orbit and ocular adnexa. Similar *MYD88* mutations are frequent in non-orbit and ocular adnexal B-cell neoplasms, including Waldenström's macroglobulinemia (79–100%) and diffuse large B-cell lymphomas (6–39%), but remain uncommon in marginal zone lymphomas (4–15%).[35,44–46] Among low-grade B-cell lymphomas, the L265P *MYD88* mutation is frequently associated with lymphoplasmacytic lymphoma (of which Waldenström's macroglobulinemia is an IgM-producing subtype).[47] In our cohort of *MYD88* mutant marginal zone lymphoma cases, lymphoplasmacytic lymphomas were excluded in each case on morphologic, immunophenotypic, and clinical grounds. No patient demonstrated the presence of urinary or serum paraprotein, no amyloid deposition was present, and CD38 expression (dim) was observed in only one case. Although lymphoplasmacytic lymphomas have been rarely described in the orbit and ocular adnexa, nearly all of these cases have been preceded by a history of lymphoma/Waldenström's macroglobulinemia or were known to have widespread disease at the time of ocular involvement.[48,49] Importantly, all of our marginal zone lymphoma cases with *MYD88* mutations were isolated primary ocular tumors. Plasmacytic differentiation has been described frequently in ocular marginal zone lymphomas[6,48,50] and was noted by our hematopathologists in varying degrees among our *MYD88* mutant marginal zone lymphomas. Indeed, the possible correlation between plasmacytic differentiation and *MYD88* mutations is an intriguing topic for future, expanded studies of marginal zone lymphomas of the orbit and ocular adnexa.

MYD88 mutations have been reported infrequently (0–7%) in non-lymphoplasmacytic lymphomas and extranodal marginal zone lymphomas,[41,46,47,51–53] the most common form (76–100%) of orbital and ocular adnexal lymphomas.[41,46,47,51,54] Hence, the observed rates of *MYD88* mutations in our marginal zone lymphomas (25%) and diffuse large B-cell lymphomas (71%) are notably higher than those previously reported in NHLs outside the orbit and ocular adnexa. Interestingly, all *MYD88* mutant diffuse large B-cell lymphomas of the orbit and ocular adnexa were of the non-germinal center B-cell (activated B-cell) subtype, consistent with previous reports that link *MYD88* alterations to activated B-cell diffuse large B-cell lymphoma subtypes in extranodal NHLs outside the orbit and ocular adnexa.[45]

The fact that lymphomas in the orbit and ocular adnexa have different rates of somatic alterations than lymphomas outside of this region is not new. For instance, *MALT1/IGH* translocations are relatively rare in marginal zone lymphomas of the orbital and ocular adnexal region when compared with marginal zone lymphomas outside this location.[55] The distinct frequency of genetic lesions such as *MYD88* mutations and *MALT1/IGH* rearrangements in our cohort of diffuse large B-cell lymphomas and marginal zone lymphomas versus similar subtypes in lymphomas outside the orbit and ocular adnexa may suggest that additional mechanisms or selection events are involved during lymphomagenesis in the orbit and ocular adnexa, a unique extranodal site. In addition, as our panel did not include *MALT1/IGH* rearrangements, we were unable to assess whether an association with this rearrangement and *MYD88* alterations exists, or whether these are mutually exclusive in diffuse large B-cell lymphomas and marginal zone lymphomas of the orbit and ocular adnexa. More research with a larger number of orbital and ocular adnexal lymphomas will be important for confirmation of these findings, assessment of whether *MALT1/IGH* rearrangements occur jointly or independently with *MYD88* mutations, and for investigation of the potential unique roles

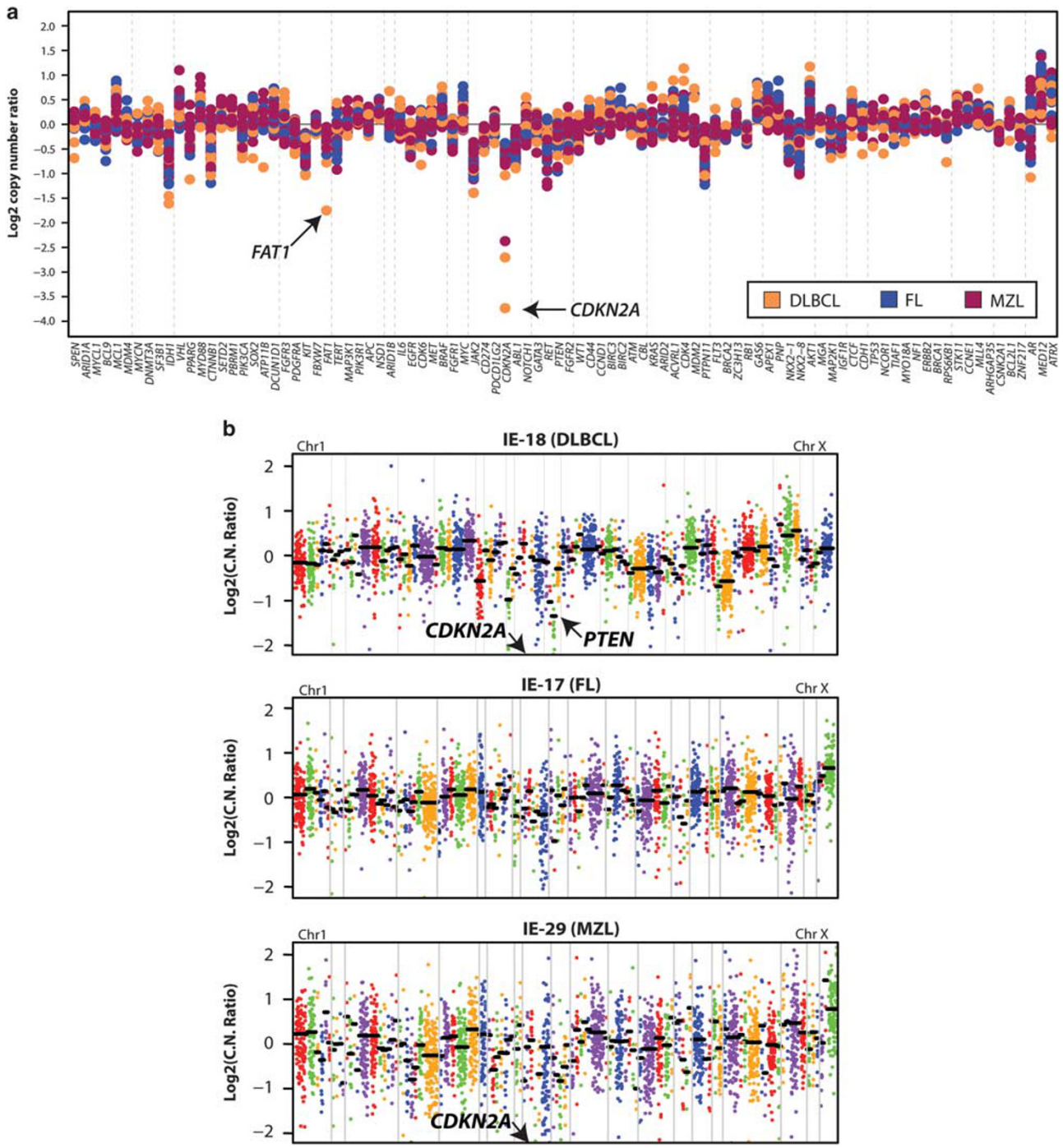


Figure 3 Copy number analysis of orbital and ocular adnexal lymphomas from next-generation sequencing (NGS) data. For each sequenced orbital and ocular adnexal lymphoma, GC-content corrected, normalized read counts per amplicon were divided by those from a composite normal sample, yielding a tumor-to-normal copy number ratio for each amplicon. Gene-level copy number estimates were determined by taking the weighted mean of the per-probe copy number ratios. (**a**) Summary of gene-level copy number ratios ($\log_2$) for all profiled orbital and ocular adnexal lymphomas stratified by subtype according to the legend. Selected genes of interest with high-level copy number alteration are indicated. Copy number plot for IE-23 was not informative and was removed from Figure 3a. (**b**). Copy number profiles for three individual orbital and ocular adnexal lymphomas, IE-18 (diffuse large B-cell lymphoma, DLBCL), IE-17 (follicular lymphoma, FL), and IE-29 (marginal zone lymphoma, MZL). Log_2 tumor-to-normal copy number ratios per amplicon are plotted (with each individual amplicon represented by a single dot and each individual gene indicated by different colors), with gene-level copy number estimates (black bars) determined by taking the weighted mean of the per-probe copy number ratios. Selected high-level copy number alterations are indicated. Log_2 copy number ratios for *CDKN2A* in both IE-18 and IE-29 are off the scale (IE-18 = – 4.26; IE-29 = – 2.37). IE-17 shows no copy number changes.

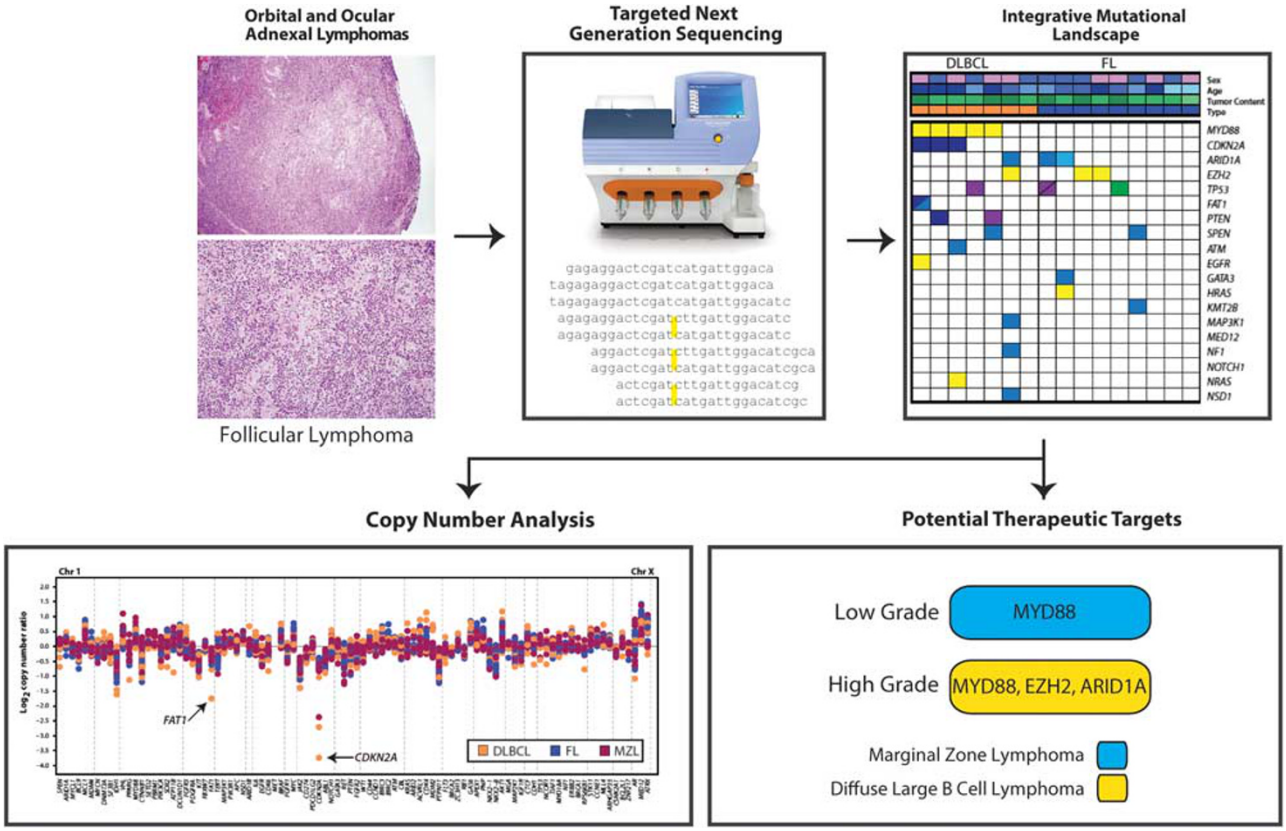


Figure 4 Workflow of determining driver and potentially actionable genomic alterations. Genomic DNA from formalin-fixed, paraffin-embedded orbital and ocular lymphoma tissue enriched by macrodissection is subjected to targeted next-generation sequencing (NGS) using a cancer gene panel. Bioinformatics analysis yields candidate point mutations, small indels and copy number alterations that potentially drive tumor growth and development in orbital and ocular adnexal lymphomas. Potential therapeutic targets are prioritized and reported.

these genetic lesions play in the etiology of these tumors in this site.

Importantly, in addition to numerous clinical trials targeting downstream MYD88-dependent factors in the NF-κB signaling pathway (such as phosphorylated Bruton tyrosine kinase and interleukin-1 receptor-associated kinases 1 and 4), and those stratifying response by *MYD88* p.L265P mutation status, currently recruiting clinical trials in diffuse large B-cell lymphomas assessing TLR inhibitors require the *MYD88* p.L265P mutation as an entry criterion (https://clinicaltrials.gov/ct2/results?term=myd88; accessed 23 August 2015). In summary, our results support evaluation of therapeutic strategies targeting MYD88 and downstream mediators activated by gain-of-function mutations in diffuse large B-cell lymphomas and marginal zone lymphomas of the orbit and ocular adnexa.

Alterations in chromatin-modifying genes such as those that encode histone lysine methyltransferases (eg, *KMT2B*, *KMT3B*, and *EZH2*) and ATP-dependent chromatin remodelers (eg, *ARID1A*) are among the most common mutations in human cancers, including many forms of B-cell lymphomas, especially diffuse large B-cell lymphomas and follicular lymphomas.[7,36–39,56–61] Indeed, in our cohort, prioritized mutations in chromatin-modifying genes were found only in diffuse large B-cell lymphomas and follicular lymphomas (6 of 16 (38%) with prioritized alterations). Specifically, 2 of 9 (22%) follicular lymphomas and 1 of 7 (14%) diffuse large B-cell lymphomas harbored prioritized *EZH2* or *ARID1A* mutations, rates that are consistent with extra-orbital and ocular adnexal diffuse large B-cell lymphomas and follicular lymphomas.[36–38,40] The single *EZH2* mutant diffuse large B-cell lymphoma case in our cohort was of the germinal center B-cell phenotype, consistent with NHLs outside the orbit and ocular adnexa.[38] Given its association with poor prognosis in several cancers, there has been intense interest in the development of EZH2 inhibitors.[62–64] At present, there are three trials (https://clinicaltrials.gov/ct2/results?term=ezh2; accessed 23 August 2015) currently recruiting individuals with relapsed or refractory B-cell lymphomas (including diffuse large B-cell lymphomas and follicular lymphomas) for evaluation of oral EZH2 inhibitors in phase 1 and 2 clinical trials. In these trials, the presence of gain-of-function *EZH2* mutations will be tested, in order to determine in which arm an affected individual will enroll (wild type or mutant *EZH2* status). Likewise, a recent report demonstrated that loss-of-function *ARID1A*

mutations in ovarian clear cell carcinomas render these tumors sensitive to EZH2 inhibitors *in vitro* and *in vivo*,[65] suggesting additional ways to target alterations in histone modifiers based on synthetic lethality. Of interest, a diffuse large B-cell lymphoma sample (IE-39) contained both a gain-of-function *EZH2* mutation (p.Y646S) and a loss-of-function *ARID1A* mutation (p.Q1363X), suggesting potentially remarkable sensitivity to EZH2 inhibition. In summary, our results support frequent potentially targetable alterations in histone/chromatin modifiers in diffuse large B-cell lymphomas and follicular lymphomas of the orbit and ocular adnexa, consistent with results in non-orbital and ocular adnexal NHLs.

In recent years, significant interest has emerged in the potential clinical value of NGS of tumoral DNA, in order to discover or guide strategies that link personalized therapies to specific genomic alterations present in the cancer. For the first time, we apply comprehensive next-generation genomic profiling to low- and high-grade formalin-fixed, paraffin-embedded orbital and ocular adnexal lymphomas, cancer subtypes for which comprehensive NGS-based profiling has not been reported. Of note, the NGS panel used herein was designed to target pan-cancer (including lymphoma) altered genes filtered to those with near-term potential actionability and a modified, lymphoma/solid tumor-specific version[26] is being used in the NCI-MATCH trial, a multi-site basket trial sponsored by the National Cancer Institute, which aims to match patients to investigational therapeutics based on their prioritized mutation profile rather than the site of tumor origin. We did not analyze non-tumor cells from the corresponding orbital and ocular adnexal lymphoma samples; thus, to distinguish germline from somatic variants, we cross-referenced candidate variants with data from the NHLBI Grand Opportunity Exome Sequencing Project (ESP6500) and the 1000 Genomes project, which include normal variants from several thousand individuals, and took into account variant fractions (for details, see Materials and Methods). Taken together, we have previously confirmed that our filtering criteria (Materials and Methods) identify prioritized high-confidence somatic variants from formalin-fixed, paraffin-embedded tissue, which pass Sanger sequencing validation with $>95\%$ accuracy, which is a nearly identical process as that used in the NCI-MATCH Trial, which also does not analyze non-tumor tissue from the corresponding lymphoma or solid tumor samples.[26,28–32]

Limitations of our study include a single site cohort, relatively few samples for each subtype, and the use of a targeted panel-based approach without assessment of other key alterations (eg, chromosomal rearrangements, methylation, and transcriptional profiling). Hence, larger, prospective, multi-institutional studies will be required to confirm our findings and clinical trials are needed in orbital and ocular adnexal lymphomas to test potential treatment strategies proposed herein.

In conclusion, through NGS targeting informative/potentially actionable genomic alterations, we identified recurrent mutations and copy number alterations across 36 orbital and ocular adnexal lymphomas, including diffuse large B-cell, follicular, and marginal zone lymphoma subtypes. There are few, if any reports, which have comprehensively profiled primary orbital and ocular adnexal lymphomas, especially diffuse large B-cell and follicular lymphomas, which is a unique feature of this study. We identified *MYD88* gain-of-function mutations at higher rates in orbital/ocular adnexal diffuse large B-cell lymphomas and follicular lymphomas (71% and 25%, respectively) than those reported in non-orbital/ocular sites; nearly all of these tumors were primary to the orbit or ocular adnexa (Figure 1c). Likewise, histone/chromatin modifiers showed frequent alterations in our cohort (at similar rates to non-orbital/ocular adnexal sites), including potentially targetable gain-of-function mutations in the histone methyltransferase *EZH2* among diffuse large B-cell lymphomas and follicular lymphomas of the orbit and ocular adnexa.

Importantly, 25% of the sequenced samples carried specific GoF mutations in *MYD88* (diffuse large B-cell and marginal zone lymphomas) and *EZH2* (diffuse large B-cell and follicular lymphomas). These specific alterations satisfy entry criteria for four ongoing clinical trials: three that use EZH2 pharmacologic inhibitors (NCT01897571, NCT02082977, and NCT02395601) in any type of B-cell lymphoma and one that uses an oligonucleotide (IMO-8400) to inhibit the activity of oncogenic L265P mutant MYD88 protein (NCT02252146) in diffuse large B-cell lymphomas. As *MYD88* mutations are even more common among diffuse large B-cell and marginal zone lymphomas of the orbit and ocular adnexa than similar subtypes outside of this region, therapies targeting this lesion may be even more effective for diffuse large B-cell and marginal zone lymphomas occurring in these locations. As novel therapeutic approaches are urgently needed for orbital and ocular adnexal lymphomas, owing to poor survival rates and high rates of relapse (diffuse large B-cell lymphomas), as well as local and vision-related toxicities of present treatments (follicular and marginal zone lymphomas), our results demonstrate the utility of a next-generation-based approach to nominate precision therapeutic approaches for orbital and ocular adnexal lymphomas, and other intraocular, ocular adnexal, and orbital tumors.

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Disclosure/conflict of interest

SAT has received travel support and had a separate sponsored research agreement with Compendia Bioscience/Life Technologies/ThermoFisher Scientific, which provides access to the sequencing panel used herein.[26] No other aspect of the study described herein was supported by Compendia Bioscience/Life Technologies/ThermoFisher Scientific. All other authors declare no conflicts of interest.

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