RESEARCH ARTICLE

MAP2K1 and *MAP3K1* Mutations in Langerhans Cell Histiocytosis

David S. Nelson,[1] Astrid van Halteren,[2] Willemijn T. Quispel,[2] Cor van den Bos,[3] Judith V.M.G. Bovée,[4] Bhumi Patel,[1] Gayane Badalian-Very,[1,5] Paul van Hummelen,[6] Matthew Ducar,[6] Ling Lin,[6] Laura E. MacConaill,[6,7] R. Maarten Egeler,[8] and Barrett J. Rollins[1,5]*

[1]Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA
[2]Immunology Laboratory, Willem Alexander Children's Hospital, Leiden University Medical Center, Leiden, the Netherlands
[3]Emma Children's Hospital Academic Medical Center, Amsterdam, the Netherlands
[4]Department of Pathology, Leiden University Medical Center, Leiden, the Netherlands
[5]Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, MA
[6]Center for Cancer Genome Discovery, Dana-Farber Cancer Institute, Boston, MA
[7]Department of Pathology, Brigham & Women's Hospital and Harvard Medical School, Boston, MA
[8]Division of Hematology/Oncology, Hospital for Sick Children, Toronto, ON

Langerhans cell histiocytosis (LCH) is now understood to be a neoplastic disease in which over 50% of cases have somatic activating mutations of *BRAF*. However, the extracellular signal-related (ERK) pathway is activated in all cases including those with wild type *BRAF* alleles. Here, we applied a targeted massively parallel sequencing panel to 30 LCH samples to test for the presence of additional genetic alterations that might cause ERK pathway activation. In 20 *BRAF* wild type samples, we found 3 somatic mutations in *MAP2K1* (*MEK1*) including C121S and C121S/G128D in the kinase domain, and 56_61QKQKVG>R, an in-frame deletion in the N-terminal regulatory domain. All three variant proteins constitutively phosphorylated ERK in in vitro kinase assays. The C121S/G128D and 56_61QKQKVG>R variants were resistant to the mitogen-activated protein kinase kinase (MEK) inhibitor trametinib in vitro. Within the entire sample set, we found 3 specimens with mutations in *MAP3K1* (*MEKK1*), including two truncation mutants, T779fs and T1481fs; T1481fs encoded an unstable and nonfunctional protein when expressed in vitro. T779fs was present in a specimen carrying BRAF V600E. The third variant was a single nucleotide substitution, E1286V, which was fully functional and is likely a germline polymorphism. These results indicate that LCH cells can harbor additional genetic alterations in the RAS-RAF-MEK pathway which, in the case of MAP2K1, may be responsible for ERK activation in a wild type *BRAF* setting. The resistance of some of these variants to trametinib may also have clinical implications for the combined use of RAF and MEK inhibitors in LCH.

INTRODUCTION

Langerhans cell histiocytosis (LCH) is a rare disease characterized by the accumulation of cells that share phenotypic features with Langerhans cells (LCs), the primary antigen-presenting cells of skin and mucosal surfaces (Bechan et al., 2006; Egeler et al., 2010; Badalian-Very et al., 2013). It has a broad spectrum of clinical presentations but all include a prominent inflammatory component. The understanding of LCH pathobiology has evolved rapidly over the past 5 years. The discovery of activating somatic mutations of *BRAF* in over 50% of LCH cases has firmly classified this disease as a neoplastic disorder (Badalian-Very et al., 2010; Sahm et al., 2012; Satoh et al., 2012; Kansal et al., 2013; Berres et al., 2014), a categorization first implied by the clonality of LCH cells (Willman et al., 1994; Yu et al., 1994). Furthermore, analysis of gene expression patterns suggests that LCH cells are more closely related to myeloid dendritic cell precursors than to LCs (Allen et al., 2010). The demonstration of mutated *BRAF* alleles in CD34+ bone marrow cells in some patients supports the characterization of LCH as a myeloid neoplasm albeit one with a strong inflammatory component (Berres et al., 2014).

Additional Supporting Information may be found in the online version of this article.

R. Maarten Egeler and Barrett J. Rollins contributed equally to this work.

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*Correspondence to: Barrett J. Rollins, MD, PhD; Department of Medical Oncology, Dana-Farber Cancer Institute, 450 Brookline Avenue, Boston, MA 02215.
E-mail: barrett_rollins@dfci.harvard.edu

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Despite the absence of detectable activating alleles of *BRAF* in 40–50% of LCH cases, LCH cells in all cases show evidence for activation of the mitogen-activated protein kinase (MAPK) signaling pathway (Badalian-Very et al., 2010). In an effort to uncover other genetic activators of this pathway, LCH tissues have been analyzed by a variety of means. For example, whole exome sequencing revealed a compound activating mutation of *ARAF* in a patient with wild type *BRAF* alleles (Nelson et al., 2014). Here, we report the presence of additional mutations in the MAPK pathway in LCH.

MATERIALS AND METHODS

LCH Samples

Samples from 23 LCH patients were obtained from the Departments of Pathology at the Leiden University Medical Center and Academic Medical Center Amsterdam in the form of formaldehyde fixed, paraffin embedded (FFPE) slides. These samples were handled according to the Dutch code of proper secondary use of human material as accorded by the Dutch Society of Pathology (www.federa.org). The samples were handled in a coded (pseudonymized) fashion according to the procedures approved by the Leiden University Medical Center ethical board. An additional 15 FFPE LCH specimen cores were obtained from the Departments of Pathology at Brigham and Women's Hospital and Boston Children's Hospital, both in Boston, MA.

Targeted Sequencing

DNA was extracted using the truEXTRAC FFPE DNA kit (Covaris, Woburn, MA) and targeted Illumina sequencing was performed as described (Nelson et al., 2014). Lane performance criteria are also described elsewhere (Nelson et al., 2014). Genomic regions in the targeted sequencing panel, known as OncoPanel v. 2.0, are listed in Supporting Information Table 1.

MAP2K1 and MAP3K1 Vectors and Mutagenesis

Vectors expressing C-terminal DDK- (FLAG-) tagged human *MAP2K1* and *MAP3K1* (purchased from Origene Technologies, Bethesda MD, catalog numbers RC218460 and RC214358, respectively) were used as templates for two-step PCR mutagenesis by primer extension. The Origene *MAP2K1* cDNA encodes a serine at position 89 instead of proline as indicated in the reference sequence (nm_002755). Therefore, a Pro89 construct was made and used as a template for subsequent mutagenesis reactions to produce MAP2K1 C121S, G128D, and C121S/G128D. Mutagenized cDNAs encoding full-length *MAP2K1* were reinserted into the pCMV6-Entry vector in which they had been supplied by Origene at 5′ AsiSI and 3′ MluI restriction sites. The *MAP2K1* in-frame deletion mutant 56_61QKQKVG>R was made by inserting a 330 nucleotide fragment (synthesized by Eurofins MWG Operon, Huntsville AL) into the vector at AsiSI and BspEI restriction sites. The *MAP3K1* E1286V mutagenized PCR product was inserted into the Origene *MAP3K1* vector at HindIII and MluI restriction sites. The *MAP3K1* single nucleotide deletion resulting in a L1481fs*8 truncation mutant was designed to allow in-frame expression of the vector encoded DDK tag eight amino acids downstream from the deletion.

In Vitro Kinase Assay

HEK293T cells were transfected with 0.5 μg DDK-tagged *MAP2K1* or *MAP3K1* cDNAs or with empty vector (pCMV6-Entry Origene # PS100001) using FuGENE HD Transfection Reagent (Promega, Madison WI). Forty-eight hours later, cells were lysed in Tris-buffered saline (TBS) containing 10% glycerol, 1% IGEPAL CA-630 (Sigma-Aldrich, St. Louis, MO), 1mM dithiothreitol, and protease inhibitors (Roche Complete, Roche, Basel Switzerland). For *MAP3K1* transfected cells the lysis buffer was supplemented with 25 mM sodium fluoride, 25 mM β-glycerophosphate, and 1 mM sodium orthovanadate. Lysates were clarified by centrifugation at 16,000 *g* for 5 min. MAP2K1 and MAP3K1 were immunoprecipitated from lysates by 90 min incubation with mouse anti-FLAG M2 antibody (Sigma-Aldrich # F3165) and Protein G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA). Immunoprecipitates were washed three times in lysis buffer and once in TBS kinase buffer containing 1 mM dithiothreitol, 10 mM magnesium chloride, 10 mM β-glycerophosphate, and 1 mM sodium orthovanadate. The kinase reaction was initiated by adding inactive GST-MEK1 protein (Millipore #14–420, Bedford, MA) or inactive GST-MAP Kinase 1/ERK2 protein (Invitrogen # PV3314) in 50 μl kinase buffer with 0.5 mM ATP to immunoprecipitated MAP2K1 or MAP3K1. Kinase reactions were incubated at 30°C for 20 min then stopped by addition of Laemmli's SDS-Sample Buffer (Boston BioProducts, Ashland, MA) and

TABLE 1. Mutations in *BRAF*, *MAP2K1*, and *MAP3K1* in LCH samples[a]

	Mutations			
Sample no.	ARAF	BRAF	MAP2K1	MAP3K1
1				
2		V600E (0.20)		
3		V600E (0.21)		T799fs (0.13)
4		V600E (0.10)		
5				
6				
7		V600E (0.12)		
8			C121S/G128D (0.09/0.10)	
9				L1481fs (0.37)
10				
11		V600E (0.15)		
12				
13[b]	Q347_A348del/F351L (0.15)[c]			
14				
15		V600E (0.33)		
16[b]		V600E (0.39)		
17			Q56_G61del>R (0.20)	
18				
19				
20				
21				E1286V (0.48)
22				
23		V600E (0.04)		
24		V600E (0.16)		
25				
26			C121S (0.20)	
27				
28				
29				
30		V600E (0.22)		

[a]LCH samples were analyzed using a targeted massively parallel DNA sequencing panel as described in the text. The allele fraction for each variant is shown in parentheses.
[b]These samples and their *BRAF* and *ARAF* allele status were reported previously (Nelson et al., 2014).
[c]Allele fraction for F351L only.

heating at 95°C. Samples were resolved on precast 4–15% gradient SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) and transferred electrophoretically to polyvinylidine fluoride (PVDF) membranes. Membranes were probed with the following antibodies: anti-phospho-MEK1/2 (Cell Signaling # 9121S, Cell Signaling, Beverly, MA), anti-p44/42 MAPK (ERK1/2) (Cell Signaling #9102), anti-phospho-p44/42 MAPK (ERK1/2) (Cell Signaling #4370S), and anti-FLAG M2. Anti-rabbit-HRP (SouthernBiotech, Birmingham, AL) and anti-Mouse-HRP (Southern Biotech) were used as secondary antibodies. Western blots were treated with chemiluminescent substrate (SuperSignal West Pico, Thermo Fisher) and signal was visualized on HyBlot CL film (Denville Scientific, South Plainfield, NJ). For drug inhibition studies the kinase reaction was carried out in kinase buffer with 1% dimethyl sulfoxide (DMSO) and varying concentrations of trametinib (Selleckchem, Houston, TX # S2673).

RESULTS

Identification of MAPK Pathway Mutations in LCH

To uncover genetic causes other than activating *BRAF* mutations for MAPK pathway activation in LCH cells, we tested 38 archived LCH samples using a multiplex targeted massively parallel sequencing panel which covers 505 genes and 15 breakpoints in common translocations (Supporting Information Table 1). Of the 38 samples originally collected for analysis, 8 were excluded for technical reasons (4 had insufficient DNA, 2 failed library preparation, 1 had unacceptably low sequencing coverage, and 1 had no $CD1a^+$ cells). Targeted sequencing results for the remaining 30 samples are shown in Table 1. The single

nucleotide variant encoding BRAF V600E was detected in 10 samples and the allele fraction in each suggested that these are somatic mutations. One sample (number 13), which contained the compound mutation in *ARAF* encoding ARAF Q347_A348del/F351L, is identical to the sample we reported previously and independently confirms the presence of this mutation in an FFPE sample which was taken from the same location as the frozen sample used earlier (Nelson et al., 2014). One of the BRAF V600E cases, sample number 16, was also reported previously (Nelson et al., 2014).

In the remaining 19 samples, which were *BRAF* and *ARAF* wild type, mutations in *MAP2K1* (MEK1) were detected in 3. These include: *MAP2K1* chr15_g.66727450CAGAAGCAGAAGG TGG>C (p.56_61QKQKVG>R), a deletion in the N-terminal regulatory domain; *MAP2K1* chr15_g.66729153T>A (p.C121S), a single nucleotide substitution in the kinase domain; and both *MAP2K1* chr15_g.66729154G>C (p.C121S) and *MAP2K1* chr15_g.66729175G>A (p.G128D). The deletion mutation has not previously been reported although it occurs in the same region of exon 2 in which short deletions have been observed in other diseases (Greger et al., 2012; Waterfall et al., 2014). The alteration encoding the C121S substitution in sample 26 has been reported previously in melanoma (Wagle et al., 2011). Informatics analysis indicated that the two alterations in the kinase domain in sample 8 occurred in *cis* (i.e., on the same chromosome). Although the amino acid substitution C121S has been reported before, the nucleotide substitution encoding it in this sample (chr15_g.66729154G>C) is novel. The nucleotide change encoding the G128D substitution has been reported previously (Emery et al., 2009; Wagle et al., 2011).

Three alterations in *MAP3K1* were observed. These include: *MAP3K1* chr5_g.56177067TG>T (p.T779fs), *MAP3K1* chr5_g.56189410TA>T (p.L1481fs), and *MAP3K1* chr5_g. 56180528A>T (p.E1286V). The third variant but neither of the first two variants appears in dbSNP. The allele fraction of the MAP3K1 E1286V single nucleotide alteration was 0.48, which was much higher than expected for a somatic variant based on LCH cell prevalence in the sample, raising a suspicion that it may be a germline variant. If expressed at all, the T779fs variant would be expected to be lacking its kinase domain (which extends from amino acids 1,243–1,508) while the L1481fs variant would retain 90% of the kinase domain.

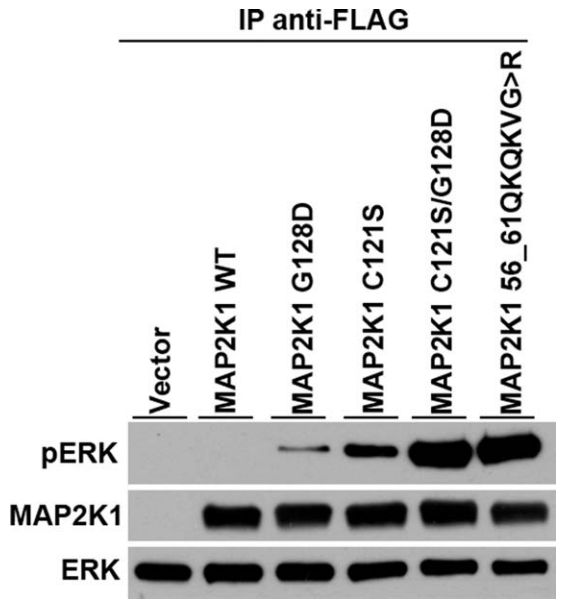

Figure 1. Kinase activities of MAP2K1 variants found in LCH cases. cDNAs encoding FLAG epitope-tagged wild type MAP2K1, MAP2K1 G128D, and the variants listed in Table 1, as well as empty vector, were transfected into HEK293T cells. Anti-FLAG immune precipitations from cell lysates were added to in vitro kinase assays using inactive synthetic ERK2 as substrate. ERK2 phosphorylation was measured by immunoblot using an anti-phospho-ERK antibody. Amounts of MAP2K1 and ERK were determined using antibodies specific for those proteins.

Kinase Activities of MAP2K1 and MAP3K1 Variants

To examine the functional consequences of these variants in MAPK pathway genes, we measured their activities in kinase assays. FLAG epitope-tagged versions of wild type MAP2K1 and the variants listed in Table 1 were expressed in HEK293T cells and anti-FLAG immune precipitates were tested for their ability to phosphorylate a synthetic ERK substrate in vitro (Fig. 1). Under the conditions used in this assay i.e., absence of activated BRAF, wild type MAP2K1 had very little detectable kinase activity. MAP2K1 G128D, which was not observed by itself in our sample set but appears alone in a mutagenized vemurafenib-resistant melanoma cell line (Wagle et al., 2011), had low but detectable kinase activity. The MAP2K1 C121S variant, which was present in sample 26, had substantial activity but the compound C121S/G128D mutant in sample 8 was many-fold more active. Its kinase activity was comparable to that of the regulatory domain deletion variant MAP2K1 56_61QKQKVG>R (sample 17).

Most of these samples were derived from anonymized discarded material and therefore detailed clinical information on the patients from whom

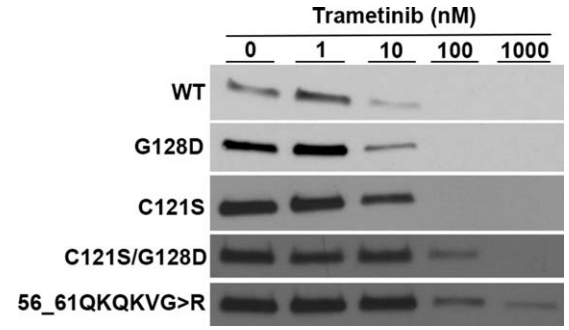

Figure 2. Sensitivity of MAP2K1 variants to inhibition by trametinib. The FLAG epitope-tagged MAP2K1 variants used in Fig. 1, as well as wild type MAP2K1, were immune precipitated using anti-FLAG and added to in vitro kinase assays as described in Fig. 1. The MEK inhibitor trametinib was added to achieve the final concentration indicated. Reactions were stopped after 20 min and the amount of ERK phosphorylation was determined by immunoblot using an anti-phospho-ERK antibody as described in Fig. 1.

the samples were taken is not available. To understand possible genotype/phenotype correlations involving mutations such as these, it will be important to plan prospectively for gathering clinical and specimen information from LCH patients. Nonetheless, we attempted to infer the clinical relevance of these variants by examining their sensitivity to the MEK inhibitor trametinib. The inhibitor was added to in vitro kinase assays at various concentrations and the intensity of phospho-ERK staining by immunoblot was used to derive semi-quantitative inhibitory potencies (Fig. 2). The IC_{50} for wild type MAP2K1 and the G128D and C121S single variants appeared comparable at 1–10 nM. Interestingly, 1nM trametinib induced a slight increase in kinase activity in wild type MAP2K1 and the G128D variant. The compound C121S/G128D variant appeared to be more resistant than either variant alone with an IC_{50} between 10 and 100 nM. The 56_61QKQKVG>R regulatory domain deletion variant was slightly more resistant.

Attempts to express the MAP3K1 frameshift mutation L1481fs resulted in the appearance of multiple protein bands below the molecular size of the expected protein suggesting that these C-terminal deletion variants are unstable (Fig. 3). Not surprisingly, the anti-FLAG immune precipitates had no detectable ERK kinase activity. In contrast, MAP3K1 E1286V from sample 21 had potent ERK kinase activity. That result plus its high allele fraction of 0.48 suggests that this is a germline polymorphism.

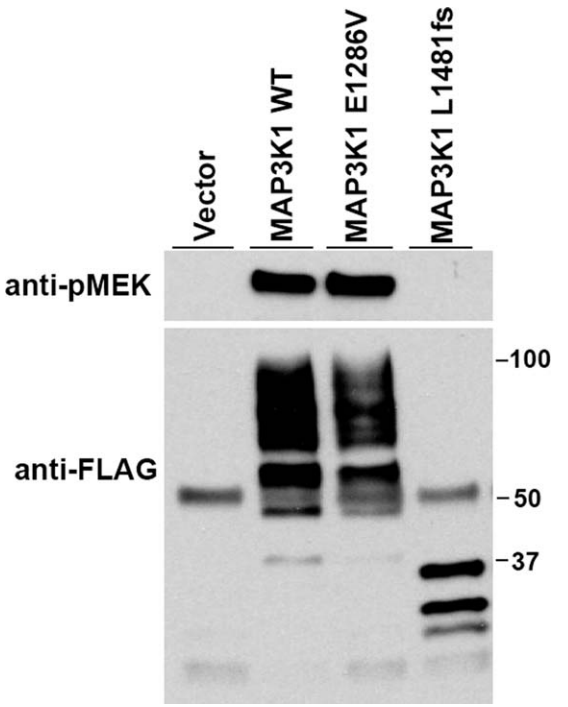

Figure 3. Expression and kinase activity of MAP3K1 variants. FLAG epitope-tagged MAP3K1 and the indicated variants were expressed in HEK293T cells, immune precipitated using anti-FLAG and added to in vitro kinase assays using inactive synthetic MEK1 as substrate. The amount of MEK1 phosphorylation was determined by immunoblot using an anti-phospho-MEK antibody (upper panel). Total FLAG epitope-containing material present in the kinase assays was determined by anti-FLAG immunoblot (lower panel). (Immunoreactive material at 55 kD is immunoglobulin heavy chain.)

DISCUSSION

The discovery of recurrent activating somatic mutations in *BRAF* has shaped our current understanding that LCH is a neoplastic disease with inflammatory features. Several studies now confirm the prevalence of these mutations at 30–60% of cases (Badalian-Very et al., 2010; Sahm et al., 2012; Satoh et al., 2012; Kansal et al., 2013; Berres et al., 2014). Because activation of the RAF-MEK-ERK signaling pathway is observed in all cases (Badalian-Very et al., 2010), additional activating mutations in other members of this cascade were expected. Our report describes three cases with *MAP2K1* mutations which we show encode constitutively active ERK kinases. Each occurs in a wild type *BRAF* context, consistent with the notion that BRAF and MAP2K1 are acting in the same transformation pathway.

These MAP2K1 mutations occur in well studied domains of the protein in which activating mutations have been described in other diseases including hairy cell leukemia (Waterfall et al., 2014). The 56_61QKQKVG>R deletion appears in the negative regulatory domain and although this particular deletion has not been described before, it overlaps other deletions in this domain which activate the kinase (Greger et al., 2012; Brown et al.,

2014; Chakraborty et al., 2014; Waterfall et al., 2014). The C121S mutation occurs in the catalytic core and was first observed in the melanoma of a patient whose disease had become resistant to vemurafenib (Wagle et al., 2011). As reported by others (Wagle et al., 2011), we also find that this mutation creates a constitutively active ERK kinase. The C121S/G128D double mutation also occurs in the catalytic core. G128D had been observed in in vitro mutagenesis screens for variants conferring resistance to a MEK inhibitor (Emery et al., 2009) or a RAF inhibitor (Wagle et al., 2011) in a melanoma cell line although its activity was not tested. Our discovery of this mutation in LCH is the first description of its de novo presence in human disease. We find that it has some constitutive activity compared to the wild type kinase, but that it works in concert with C121S in the double mutant to produce activity greater than that of C121S alone. Interestingly, the nucleotide change encoding C121S in this sample is novel (chr15_g.66729154G>C) while the change encoding C121S in the sample in which it appears alone has been reported previously (chr15_g.66729153T>A). We do not yet know if the appearance of this novel SNV is somehow linked to the appearance in *cis* of the G128D variant. Notably, the compound variant is more resistant to the MEK inhibitor, trametinib, than either variant alone. Both C121S and G128V confer trametinib resistance to A375 melanoma cells (Van Allen et al., 2014). A recent report also described variability in MEK1 inhibitor sensitivity among MAP2K1 mutants and this may turn out to have important therapeutic implications (Chakraborty et al., 2014).

Two recently published reports have described frequent MAP2K1 mutations in a cohort of LCH samples (Brown et al., 2014; Chakraborty et al., 2014). Brown et al. found in-frame deletions in the N-terminal regulatory domain, none of which were identical to the one we describe here, as well as single nucleotide substitutions in the kinase domain. As in our LCH set, one sample contained a double mutation in the kinase domain, C121S/G128V. But unlike the double mutation we described, this one contained the previously reported SNV encoding C121S and the substitution at position 128 was valine rather than aspartate. G128V has been found by itself in relapsed melanoma (Van Allen et al., 2014). It is also notable that this recent report found MAP2K1 mutations in about half of the *BRAF* wild type samples. In contrast, we observed MAP2K1 mutations in only 3 out of 21 wild type *BRAF* samples or, perhaps more properly, 3 out of 20 wild type *BRAF* or *ARAF* samples. The other recent report found 4 in-frame deletions in the N-terminal regulatory domain, again not identical to the one we described, and two in-frame deletions in the kinase domain (Chakraborty et al., 2014). Although they also described a single nucleotide variant in the N-terminal regulatory domain they saw none of the SNVs reported by Brown et al. (Brown et al., 2014) or by us. The prevalence of MAP2K1 mutations in their LCH sample set was 7 out of 41, or 17%, a frequency closer to the one we observed. The diversity of MAP2K1 mutations is quite striking given the relatively small sample set created by combining all three of these reports (ours plus those of Chakraborty et al. (Chakraborty et al., 2014) and Brown et al. (Brown et al., 2014)). A larger number of samples will have to be sequenced before the true prevalence of individual MAP2K1 mutations in LCH is known.

The mutations in MAP3K1 are more difficult to interpret. MAP3K1 phosphorylates MAP2K4 (Yan et al., 1994; Siow et al., 1997) and MAP2K7 (Hirai et al., 1998) which, in turn, phosphorylate JNK and thereby promote cell survival. MAP3K1 can also promote proliferation and survival via phosphorylation of MAP2K1 which then activates ERK signaling (Lange-Carter et al., 1993). However, the two mutants we found that are unlikely to be germline polymorphisms because of their low allele fraction (T799fs and L1481fs) appear to be loss-of-function variants. This is consistent with the frequent occurrence of MAP3K1 frameshift and other loss-of-function mutations in luminal breast cancers (Cancer Genome Atlas, 2012; Ellis et al., 2012). The basis for MAP3K1's apparent tumor suppressor activity is unclear but may be related to the fact that caspase cleavage generates a C-terminal fragment containing the kinase domain which strongly promotes apoptosis via the JNK pathway (Widmann et al., 1998). It will be important to examine the phosphorylation status of a wide sample of MAP3K1 substrates in LCH samples harboring mutations in this gene. On balance, deletion or inactivation of MAP3K1 might provide a survival advantage for cancer cells, perhaps incremental to the effect of BRAF V600E as in sample number 3. In fact, the co-occurrence of these mutations in sample 3 raises the possibility that the presence of MAP3K1 mutations may modify the response of BRAF V600E-containing LCH cells to RAF inhibitors.

Our results further define the landscape of genomic abnormalities in LCH. The presence of

activating *BRAF* mutations in LCH has led to the initiation of clinical trials testing the efficacy of BRAF inhibitors and early results look promising (Haroche et al., 2013). As in other BRAF-mutant diseases, combined treatment with RAF and MEK inhibitors is being considered. The presence in LCH of activating mutations in MAP2K1 that are relatively insensitive to MEK inhibitors would predict that some patients will have de novo resistance to this combination and sounds a note of caution.

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