Genome-wide siRNA screen for mediators of NF-κB activation

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Contributed by Elliott Kieff, December 16, 2011 (sent for review October 2, 2011)

Although canonical NFκB is frequently critical for cell proliferation, survival, or differentiation, NFκB hyperactivation can cause malignant, inflammatory, or autoimmune disorders. Despite intensive study, mammalian NFκB pathway loss-of-function RNAi analyses have been limited to specific protein classes. We therefore undertook a human genome-wide siRNA screen for novel NFκB activation pathway components. Using an Epstein Barr virus latent membrane protein (LMP1) mutant, the transcriptional effects of which are canonical NFκB-dependent, we identified 155 proteins significantly and substantially important for NFκB activation in HEK293 cells. These proteins included many kinases, phosphatases, ubiquitin ligases, and deubiquinating enzymes not previously known to be important for NFκB activation. Relevance to other canonical NFκB pathways was extended by finding that 118 of the 155 LMP1 NF-κB activation pathway components were similarly important for IL-1β–, and 79 for TNFα–mediated NFκB activation in the same cells. MAP3K8, PIM3, and six other enzymes were uniquely relevant to LMP1-mediated NFκB activation. Most novel pathway components functioned upstream of IκB kinase complex (IKK) activation. Robust siRNA knockdown effects were confirmed for all mRNAs or proteins tested. Although multiple ZC3H-family proteins negatively regulate NFκB, ZC3H13 and ZC3H18 were activation pathway components. ZC3H13 was critical for LMP1, TNFα, and IL-1β NFκB-dependent transcription, but not for IKK activation, whereas ZC3H18 was critical for IKK activation. Down-modulators of LMP1 mediated NFκB activation were also identified. These experiments identify multiple targets to inhibit or stimulate LMP1-, IL-1β–, or TNFα–mediated canonical NFκB activation.

lymphoma | inflammation | signaling | oncogene | cytokine

NFκB transcription factors are homodimers or heterodimers of REL homology domain proteins NFκB1 (p50), NFκB2 (p52), RelA, RelB, or cREL. Before stimulation, NFκB complexes are retained in the cytoplasm through association with inhibitors (IκB; for review, see ref. 1). NFκB can be activated by canonical and noncanonical pathways. Canonical NFκB signal transduction activates the IκB kinase complex (IKK), a heterotrimer of kinases CHUK (IKKα) and IKBKB (IKKβ) with the scaffold IKBKG (IKKγ). IKKγ dependence is a hallmark of canonical NFκB activation (2). IKKβ phosphorylates NFKBIA (IκBα), NFKBIB (IκBβ), and NFKBIE (IκBε), triggering their proteasomal degradation and NFκB nuclear translocation. RelA/p50 is the prototypical canonical NFκB complex. RelA phosphorylation and acetylation potentiate transcription activation (1). NFκB activity is tightly controlled by negative regulators, such as IκBs and TNFAIP3 (A20), which are among the most robustly NFκB up-regulated proteins (1, 3).

TNFα and IL-1β are cytokines that potently activate canonical NFκB and MAPK pathways, thereby mediating inflammatory and immune responses. TNFα induces TNF receptor 1 (TNFR1) trimerization and recruitment of TNFR1-associated death-domain protein (TRADD) and receptor-interacting protein kinase 1 (RIPK1). TRADD engages TNFR-associated factor 2 (TRAF2), which recruits the ubiquitin (Ub) E2 ligase UBC5 and the E3 ligases cIAP1 and cIAP2. CIAP1/2 polyubiquitinate RIPK1 and TRAF2, which recruit and activate the K63-Ub binding proteins TAB1, TAB2, and TAB3, as well as their associated kinase MAP3K7 (TAK1). TAK1 in turn phosphorylates IKKβ activation loop serines to promote IKK activity (4). The E3 ligase linear Ub chain-assembly complex (LUBAC) ubiquitinates IKKγ and RIPK1 to stabilize TNFR1 signaling complexes (5–7).

IL-1β stimulates IL-1 receptor1 (IL-1R1) assembly with the IL1RAcP accessory protein. IL-1R1 then recruits the adaptor myeloid differentiation primary response gene 88 and the kinases IRAK1, IRAK2, and IRAK4. IRAK4 phosphorylates IRAK1 and IRAK2, which recruit and oligomerize TRAF6, together with the E2 enzyme UBC13/UEV1A. TRAF6 attaches K63-Ub chains to IRAK1 and TAK1 (8). Unanchored K63-Ub chains further activate TAK1 (9). K63 and linear Ub chains (10) recruit IKKγ and stimulate IKK activity.

Epstein–Barr virus (EBV) latent membrane protein 1 (LMP1), Kaposi's sarcoma herpesvirus vFLIP, and human T-cell leukemia virus TAX are virus-encoded oncogenes that activate NFκB. In hosts with T-cell immune deficiency, EBV is a major cause of lymphoproliferative diseases. Lymphomas in HIV-infected people are frequently EBV-infected cells (11, 12). With age, EBV-immune T-cell senescence wanes, and EBV-positive diffuse large B-cell lymphoma occur in people over age 70 (13). EBV and LMP1 are also implicated in Hodgkin lymphoma and anaplastic nasopharyngeal carcinoma (NPC) (14, 15). NPC comprises 20% of cancers in southern China (16).

LMP1 constitutively aggregates in the plasma membrane and juxta-membrane endosomes (14). LMP1 activates NFκB through cytoplasmic transformation effect sites 1 (TES1) and 2 (TES2). LMP1 TES1 and TES2 are required for efficient B-lymphocyte transformation (17). EBV-transformed lymphoblastoid cells depend on NFκB for survival and die after NFκB blockade (17). LMP1 TES1 and TES2 preferentially activate noncanonical and canonical NFκB pathways, respectively (17). Indeed, LMP1 TES2 signaling is highly IKKγ-dependent (18), and nearly all LMP1 TES2 transcriptional effects can be blocked by IκBα super-repressor expression (19). The experiments reported here first used LMP1 TES2 signaling to identify HEK293 cell proteins important

Author contributions: B.E.G., E.D.C.-M., and E.K. designed research; B.E.G., N.P.S., K.T., and B.Z. performed research; B.E.G., N.P.S., B.Z., and E.D.C.-M. contributed new reagents/analytic tools; B.E.G., F.T., J.C.M., N.P.S., B.Z., J.Q., R.J.X., and E.K. analyzed data; and B.E.G., F.T., J.C.M., R.J.X., and E.K. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1120542109/-/DCSupplemental.

for canonical NFκB activation, because of LMP1's relevance to EBV-associated epithelial and lymphoid malignancies (20).

Results

Genome-Wide Screen for LMP1 NFκB Activation Mediators. Mediators of LMP1 TES2 canonical NFκB activation were identified using a TES1-null mutant LMP1 (P_{204}A and Q_{206}A), hereafter referred to as LMP1. HEK293 cells with inducible LMP1 expression (19) were transfected with siRNAs and grown for 3 d, to allow sufficient time for RNA and protein depletion. LMP1 was then expressed for 16 h, and NFκB activity was measured by flow cytometry using a stably integrated NFκB-dependent GFP reporter. Screen conditions were optimized for signal versus background with RelA-specific versus control siRNAs. The screen cell line was further validated by finding that LMP1 induced IκBα S32 and S36 phosphorylation, IκBα degradation, and RelA S276 and S536 phosphorylation (19). Knockdown of either TRAF6 or IKKγ rendered cells refractory to LMP1 induction of NFκB-dependent GFP (*SI Appendix*, Fig. S1), as anticipated (18, 21, 22). The Dharmacon siArray library of 21,121 SmartPools, with four siRNAs per mRNA target, was then used in a human genome-wide screen. *Z*-scores from replicate plates were averaged and median-adjusted SDs were determined. Screen data were highly concordant among plate replicates (*SI Appendix*, Fig. S1).

Multiple LMP1 NFκB activation pathway proteins were identified, the knockdown of which significantly decreased NFκB-dependent GFP expression (Fig. 1). Overall, 140 hits had a *z*-score < −2.5, 213 scored between −2 and −2.5, and 537 scored between −2 and −1.5 (Fig. 1). These 890 potential LMP1 NFκB activation pathway components included IKKγ ($z = -4.2$), TRAF6 ($z = -3.4$), RelA ($z = -3.4$), IKKα ($z = -2.9$), TAB1 ($z = -1.9$), TAK1 ($z = -1.8$), and UBC13 ($z = -1.6$). TAB2 or TAB3 knockdowns mildly impaired LMP1 NFκB activation ($z = -1.1$ and -1.3, respectively), consistent with partially redundant roles in K63-Ub chain binding and TAK1 activation.

Validation of LMP1 NFκB Activation Pathway Hits. To focus on mRNA-encoding proteins likely to be specifically relevant to LMP1-mediated NFκB activation, siRNA pools that targeted pseudogenes, RNA polymerase subunits, RNA splicing or transport factors, translation initiation or elongation factors, ribosome subunits, or proteasome subunits were not further studied. Similarly, siRNA effects that reduced cell number by >50% of the plate average were not further pursued because NFκB inhibition did not affect HEK293 cell growth. The remaining 349 activation pathway hits were further evaluated in duplicate, using Tet-On LMP1 293 cells with integrated NFκB-dependent GFP and NFκB-independent Tet-On DsRed reporters. Following 3 d of knockdown, LMP1-dependent GFP and LMP1-independent DsRed were induced for 16 h and quantified by FACS. Applying a Benjamini–Hochberg adjustment, 239 (68%) of the 349 siRNA pools significantly differed in GFP/DsRed ratios from control siRNAs ($P < 0.01$).

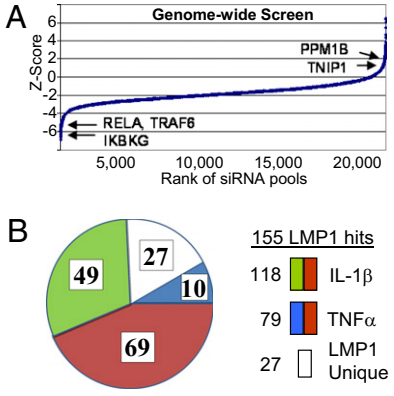

Fig. 1. Genome-wide siRNA screen for LMP1 NFκB activation pathway proteins. (*A*) Primary screen siRNA pool results are shown in mean *z*-score rank order, from decreased NFκB to increased NFκB. Exemplary known negative or positive NFκB components are indicated. (*B*) The 155 validated LMP1 NFκB activation-pathway components were tested for roles in TNFα– or IL-1β–mediated NFκB activation. One-hundred eighteen proteins were important for IL-1β– and 79 for TNFα–mediated NFκB activation. Components common to LMP1, IL-1β, and TNFα are red, to LMP1 and IL-1β are green, to LMP1 and TNFα are blue, and unique to LMP1 are indicated by white.

The 239 LMP1 activation pathway hits were further evaluated by testing each of the four individual SmartPool siRNA components in triplicate. Applying an adjusted $P < 0.01$ cutoff for at least two siRNAs per mRNA target and a >40% effect on NFκB-induced GFP expression for at least one siRNA, 178 hits were further validated. Of these hits, 23 were not further pursued because of control DsRed effects, leaving 155 proteins that were specifically important for LMP1 induced NFκB activation. The full dataset is presented in Dataset S1. NFκB pathway component expression is frequently NFκB regulated, and RNAs encoding 37 of the 155 proteins were significantly LMP1 NFκB regulated in 293 cells at a $P < 0.01$ cutoff (*SI Appendix*, Fig. S2) (19).

NFκB Activation Pathway Component Expression in Immune Cells. Because LMP1 activation of gene expression in both epithelial cells and lymphocytes is biologically relevant, the Immunological Genome project database (23) was used to explore expression of the 155 activation pathway components in B cells, T cells, dendritic cells, natural killer cells, and macrophages. Most components were highly expressed in immune cells (*z*-value > 1.96, 95% confidence). These data are available online at http://ccibweb1.mgh.harvard.edu:8080/NFkB/. LMP1 activation pathway components were significantly enriched for expression in dendritic cells (adjusted $P < 0.01$) and T cells (adjusted $P < 0.02$) (Dataset S2).

NFκB Mediators Tend to Be Enzymes or Enzyme Complex Components. By gene ontology (GO) molecular function analysis, 52 activation pathway proteins are enzymes or components of enzyme complexes (*SI Appendix*, Fig. S3 and Dataset S3). The major (adjusted $P < 0.08$) enriched enzyme categories were transferases (13%), hydrolases (13%), and kinases (8%). By GO biological process classification, activation pathway components were most enriched for metabolic process (39 components, adjusted $P < 0.004$) (*SI Appendix*, Fig. S4 and Dataset S4). These components were significantly up-regulated in immune cells (adjusted $P < 0.0001$) (Dataset S2). By KEGG analyses (24), proteins important for LMP1 NFκB activation were enriched for Toll-like, retinoic acid inducible-gene 1-like, and for nucleotide oligomerization domain-like receptor and MAP kinase signaling pathway components ($P < 0.05$).

Proteins Important for LMP1 Induced IκBα Degradation. Enzyme components were tested for their role in LMP1-mediated IκBα degradation using a stably expressed IκBα-*Photinus* luciferase fusion protein and a Renilla luciferase control reporter in HEK293 cells (25). LMP1 expression, TNFα addition, or IL-1β addition caused IκBα-*Photinus* degradation without affecting Renilla levels. Depletion of IKKγ, TRAF6, UBC13 (the K63-Ub specific E2), or TAB2 and TAB3 (K63-Ub binding adaptor proteins), significantly impaired LMP1 induced IKK activation and impaired LMP1 mediated IκBα-*Photinus* degradation ($P < 0.01$), as expected for canonical NFκB activation pathway proteins that are upstream of IκBα degradation (*SI Appendix*, Fig. S5).

Surprisingly diverse Ub ligases were also important for IκBα-*Photinus* degradation, including the CUL 4 ligase subunit DDA1,

ring-finger proteins RNF11 and RNF34, and F-box protein FBX041 (*SI Appendix*, Fig. S5). RNF34 is implicated in NFκB activation and an RNF11 complex with phosphorylated TAX1BP1, Itch, and A20 is implicated in regulating NFκB activation (26). Knockdowns of RNF11 or RNF34 increased IκBα-*Photinus* levels >50%, indicating that RNF11 and RNF34 are upstream of IκBα-*Photinus* turnover. In contrast, depletion of RNF141 increased IκBα-*Photinus* <25% and of RNF112 decreased IκBα-*Photinus* <25%, indicating that their more substantial siRNA knockdown effects on NFκB activation are likely downstream of IκBα turnover.

LUBAC is implicated in TNFα, IL-1β, LPS, and CD40-mediated canonical NFκB activation (6, 27), and siRNAs against individual subunits nearly met the LMP1 primary screen cutoff. The importance of LUBAC for LMP1-mediated NFκB activation was evident from combined RNF31 and RBCK1 knockdown, which blocked LMP1-induced NFκB GFP up-regulation and nearly doubled IκBα-*Photinus* levels (*SI Appendix*, Fig. S5).

Although deubiquitinating enzymes (DUBs) can negatively regulate NFκB (28), four DUBs were activation pathway components. USP11 siRNAs were nearly as effective as TRAF6 siRNAs in stabilizing IκBα-*Photinus* following LMP1 expression (*SI Appendix*, Fig. S5). siRNAs against USP21, USPL1, and USP43 also markedly stabilized IκBα-*Photinus*, indicating that these DUBs are activation pathway components upstream of IκBα degradation (*SI Appendix*, Fig. S5). Independent USP11 or USP43 siRNAs also significantly impaired IKK in vitro kinase activity and stabilized IκBα-*Photinus* luciferase (Fig. 2 and *SI Appendix*, Fig. S5); their knockdown by independent siRNAs was evident by Western blot and real-time PCR (*SI Appendix*, Fig. S6). USP11 associates with RelB and other NFκB pathway components (29, 30) and may increase NF-κB activation by removing degradative Ub chains from activation pathway components. USP43 associates with 14-3-3 proteins and may regulate 14-3-3–associated kinases, phosphatases, or receptor stabilities to increase or prolong NFκB activation (31). Moreover, USP21 association with protein phosphatases and microtubule affinity-regulating kinases (31) may alter membrane protein transport or cytoskeleton interactions that increase or stabilize NFκB activation.

Kinases important for IκBα-*Photinus* degradation were also identified (*SI Appendix*, Fig. S5). IKKα or IKKβ knockdowns partially stabilized IκBα-*Photinus*, and their combined depletion resulted in threefold increased IκBα-*Photinus* levels following LMP1 expression (*SI Appendix*, Figs. S5 and S7). Similar IKKα and IKKβ partial redundancies are evident in TNFα–mediated NFκB activation in HeLa cells (32) and in diffuse large B-lymphoma cells treated with an IKKβ small-molecule antagonist

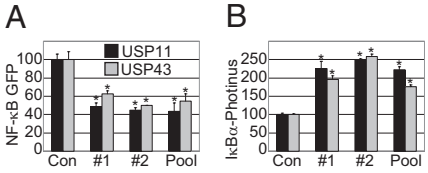

Fig. 2. USP11 and USP43 importance for LMP1-mediated IKK activation. (*A*) Effects of independent USP11 or USP43 siRNAs or siRNA pools on NFκB induction. 293 cells were induced for Tet-On LMP1 and DsRed expression. NFκB GFP and control DsRed levels were normalized to nontargeting siRNA control (Con) transfected cell levels. (*B*) Effects of USP11 or USP43 depletion on IκBα stability. 293 cells with IκBα-*Photinus* luciferase fusion protein and control Renilla luciferase expression were transfected with the indicated siRNAs. siRNA control treated cell levels were defined as 100%. Values above 100% indicate impaired degradation of IκBα-*Photinus* luciferase, attributable to decreased IKK activity. Mean and +1 SD of triplicate data are representative of at least three independent experiments. Asterisk indicates paired *t*-test $P < 0.01$ relative to siRNA control.

(25). Interestingly, siRNAs to PIM3, PKN3, or RIPK4 substantially suppressed LMP1 mediated NFκB activation and were similar to TAK1 siRNAs in these effects (*SI Appendix*, Fig. S8 and Dataset S1). RIPK4 and PKN1 interact with TRAFs (33), consistent with a cytoplasmic RIPK4 or PKN3 role in NFκB activation through TRAF phosphorylation. PIM3 depletion partially stabilized IκBα-*Photinus*, consistent with roles upstream and down stream of IκBα-*Photinus*. Although MAP3K8 (TPL2) and STK40 were significant for LMP1 NFκB activation, their depletion did not increase IκBα-*Photinus* levels, consistent with effects downstream of IκBα degradation (*SI Appendix*, Fig. S5).

Although phosphatases negatively regulate canonical (34), phosphatase catalytic subunits, PTPRS, PPP1CB, and PPM1M, and regulatory subunits PPP2R5E, PPP1R16B, and PPP4R4 were instead important for LMP1–mediated NFκB activation (Dataset S1). Depletion of PTPRS, PPP1CB, PPM1M, PPP2R5E, PPP1R16B, or PPP4R4 increased IκBα-*Photinus* levels, indicative of roles upstream of IκBα degradation (*SI Appendix*, Fig. S5). Depletion of the lipid-raft associated phosphatidic acid phosphatase, PPAP2B, also revealed a substantial positive effect on NFκB activation, as described for WNT signaling (35), but had minimal effect on IκBα-*Photinus* degradation, consistent with a role down stream of IκBα degradation. Depletion of the protein phosphatase 4 catalytic subunit, PPP4C, also did not stabilize IκBα-*Photinus*, consistent with its nuclear localization and a role downstream of IκBα degradation (36) (*SI Appendix*, Fig. S5).

ZC3H zinc-finger family RNA binding proteins control gene expression posttranscriptionally (37). Although ZC3H proteins have substantial negative effects on NFκB activation by destabilizing mRNAs encoding activation pathway components (38) and by additional mechanisms, ZC3H13 and ZC3H18 were important for LMP1-mediated NFκB activation (Dataset S1). Six different ZC3H13 siRNAs and five different ZC3H18 siRNAs had significant and substantial effects on LMP1 and TNFα, and for ZC3H13 also on IL-1β–mediated NFκB activation (Fig. 3). Knockdowns were verified by Western blot and real-time PCR (*SI Appendix*, Fig. S6). ZC3H18 depletion substantially diminished LMP1-mediated IKK activation by in vitro kinase and endogenous IκBα phosphorylation assays (*SI Appendix*, Fig. S9), and more than doubled IκBα-*Photinus* levels (Fig. 3), indicative of a major upstream role in IκBα degradation. In contrast, ZC3H13 depletion partially decreased LMP1-induced IκBα-*Photinus*, consistent with a major ZC3H13 role downstream of IκBα degradation. LMP1 upregulates ZC3H13 RNA by 1.9-fold, consistent with the observation that NFκB pathway components are often transcriptionally NFκB-regulated (19).

Brain expressed, X-linked (BEX) family adaptor proteins bind to the p75 neurotrophin receptor (NTR) cytoplasmic tail and modulate nerve growth-factor signaling pathways (39, 40). BEX2 and BEX3 (NGFRAP1) mediate p75 NTR NFκB activation, and BEX1 inhibits neurotrophin signaling by competing with RIP2 for receptor binding (40, 41). Surprisingly, BEX3 (NGFRAP1) siRNAs strongly inhibited LMP1-mediated NFκB activation and stabilized IκBα-*Photinus*, consistent with an upstream role in LMP1-induced NFκB activation (*SI Appendix*, Fig. S10). Moreover, BEX1 siRNAs enhanced LMP1-mediated NFκB by >40%. BEX1, BEX3, and BEX5 coimmunoprecipitated with LMP1 in overexpression experiments and interacted with LMP1 by split-yellow fluorescence protein two-hybrid analysis (*SI Appendix*, Fig. S10).

Importance in TNFα– and IL-1β–Induced NFκB Activation. The role of the 155 LMP1 activation pathway proteins in TNFα– and IL-1β–induced NFκB activation was assessed in 293 cells with the integrated NFκB-dependent GFP reporter. Following knockdown by each by four individual siRNAs in triplicate, cells were stimulated by TNFα (1 ng/mL) or IL-1β (25 ng/mL), and NFκB-dependent GFP expression was assayed 15 h later. Again using the criteria of $P < 0.01$ for two siRNAs and GFP reduction of >40%

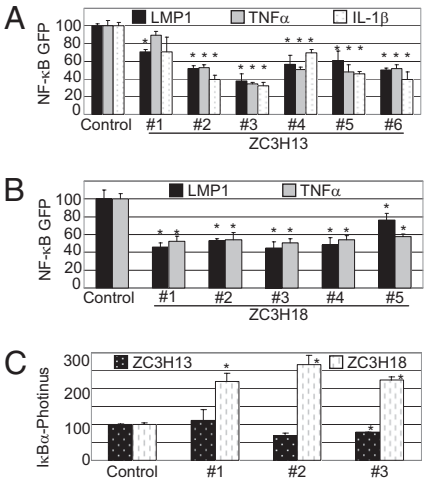

Fig. 3. ZC3H13 and ZC3H18 mediate canonical NFκB activation. Effects of individual ZC3H13 (*A*) or ZC3H18 (*B*) siRNAs on NFκB activation. ZC3H13- or ZC3H18-depleted cells were stimulated with LMP1, TNFα, or IL-1β for 15 h, and siRNA control normalized NFκB GFP reporter levels were quantitated. (*C*) ZC3H18, but not ZC3H13 depletion, impairs LMP1-mediated IκBα turnover. Median and + 1 SD of triplicate data are representative of at least three independent experiments. Asterisk indicates paired *t* test $P < 0.01$ relative to siRNA control.

for at least one siRNA, 118 proteins were important for IL-1β– and 79 for TNFα–mediated NFκB activation (Fig. 1 and Dataset S1). These proteins assembled into an extensive interactome, together with factors previously implicated in canonical NFκB activation by TNFα and IL-1β (*SI Appendix*, Fig. S11).

Of the 155 LMP1 NFκB activation-pathway proteins, 69 were important for NFκB activation by IL-1β and TNFα (Dataset S5), and are likely core canonical NFκB activation components in 293 cells. Alternatively, some proteins may be receptor trafficking components (Fig. 4). Many siRNAs that had >40% effect on LMP1-induced NFκB activation also had >40% effect on TNFα– or IL1-β–induced NFκB activation, consistent with similar importance in these three pathways (*SI Appendix*, Dataset S1). The well-established canonical NFκB pathway components TAB1, IKKγ, and RelA were important for NFκB activation by the three agonists. Interestingly, most of the other 69 proteins were newly linked to NFκB and included five kinases, three phosphatase subunits, four DUBs, three Ub ligase subunits, and the arginine methyltransferase, PRMT1 (Fig. 4). Putative scaffolds, such as the LRCH3 actin binding and leucine-rich repeat protein, were also important for NFκB activation by all three stimuli. LRCH3 associates with 14-3-3 proteins and is twofold LMP1 NFκB up-regulated in 293 cells (19). LRCH3 depletion increased IκBα-*Photinus* levels twofold in cells stimulated by LMP1, TNFα, or IL-1β, placing LRCH3 upstream of IKK activation. IL-1β–mediated NFκB activation was particularly sensitive to LRCH3 depletion, which nearly completely blocked endogenous IκBα turnover and RelA S536 phosphorylation (*SI Appendix*, Figs. S6 and S12). Multiple uncharacterized proteins were important for all three NFκB activation pathways, including the transmembrane protein TMEM101, which when overexpressed activates NFκB in HEK293 cells (42).

Proteins Uniquely Important for LMP1-Mediated NFκB. Proteins important for LMP1-mediated NFκB activation, but not for IL-1β– or TNFα–mediated NFκB activation included nine enzymes: proto-oncogenes TPL2 and PIM3, protein phosphatase PPP4C catalytic and PP4R4 regulatory subunits, three putative Ub-ligases, the sentrin peptidase SENP6, and the IKKβ substrate *N*-acetyltransferase NAA10 (Fig. 4). Because EBV-transformed cells require LMP1-mediated NFκB activation, inhibition of these enzymes may selectively block EBV effects on cell growth or survival. Notably, EBV infection up-regulates PIM kinase expression (43), and TPL2 is likewise highly expressed in EBV-associated primary Hodgkin disease and NPC cells (44). LMP1 up-regulates TPL2 expression threefold (19) and activates TPL2 kinase activity (44). Similarly, LMP1 up-regulates PPP4R4 threefold in 293 cells (19), and PPP4C interacts with p50, RelA, REL, and TRAF6 (36).

Proteins Important for LMP1 and IL-1β but Not for TNFα NFκB. Enzymes specifically important for LMP1 and IL-1β NFκB activation and not for TNFα included four Ub ligases, three kinases, and two protein phosphatase regulatory subunits (Fig. 4). IL-1β mediated NFκB activation was particularly sensitive to depletion of the protein phosphatase 4 subunit SMEK1, because siRNAs against SMEK1 reduced IL-1β–mediated NFκB activation 65–87% (Dataset S1). IL-1β–mediated NFκB activation was also more dependent on STK40 than was LMP1 or TNFα–mediated NFκB activation (*SI Appendix*, Fig. S13). LMP1 and IL-1R1 trafficking or posttranslational modification may also be affected by depletion of vesicle protein VMP1, SH3GLB2, Rho GTPase activating protein ARHGAP28, Golgi galactosyltransferase B3GNT9, or of the palmitoyltransferase ZDHHC3.

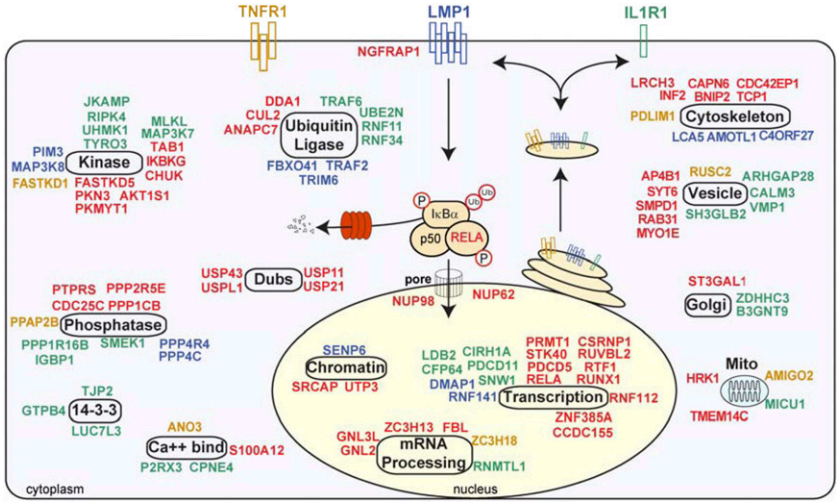

Fig. 4. Key mediators of LMP1, TNFα, and IL-1β canonical NFκB activation identified in this study, placed at positions most likely relevant to their role in NFκB activation. Our results, GO and KEGG annotations were used to position components. Colors signify importance for NFκB activation by LMP1, TNFα, and IL-1β (red), LMP1 and IL-1β (green), LMP1 and TNFα (orange), or LMP1 only (blue).

LMP1 and IL-1β activation-pathway nuclear proteins included the NFκB transcription coactivators CIRH1A (45) and PDCD11, which associates with RelA and p50 (46). The roles of these proteins in LMP1- and IL-1β–, but not TNFα–mediated NFκB activation, are worthy of further investigation.

Proteins Selectively Important for LMP1 and TNFα NFκB. Ten proteins were important for LMP1- and TNFα–, but not IL-1β–mediated NFκB activation (Dataset S5). Of these proteins, only HSP27 is previously linked to TNFα–mediated NFκB activation. HSP27 associates with the IKK complex, binds polyubiquitin chains, and enhances phosphorylated IκBα proteasomal degradation (47). The FASTKD1 kinase and PPAP2B phosphatase were required for LMP1- and TNFα–, but not IL-1β–mediated NFκB activation. The nuclear receptor NR1H4 may associate with PRMT1 to up-regulate LMP1 and TNFα NFκB-dependent transcription. The PDZ actin stress fiber binding and LIM domain protein PDLIM1, as well as the RUN and the SH3 domain protein RUSC2, are also selectively important for LMP1 and TNFR1 transport or signaling.

NFκB Inhibitors. Some siRNA pools significantly increased LMP1-mediated NFκB activation. We selected 100 of these to identify down-modulators of LMP1 induced or basal and LMP1 induced NFκB. Each of four individual siRNAs were screened for effect on basal and LMP1-induced NFκB activation. Again using the criteria of an adjusted $P < 0.01$ for two siRNAs and >40% NFκB effect for at least one siRNA (Dataset S6), 47 down-modulators of LMP1-mediated NFκB activation were identified. Validated hits included IκBα and G3BP2, a factor involved in IκBα turnover. Additional well-characterized NFκB inhibitors included the DUB CYLD, the RNase ZC3H12b, and poly-Ub binding TNIP1 (ABIN1). Each ABIN1 siRNA increased LMP1 mediated NFκB activation (Dataset S6). Consistent with ABIN1 being a feedback down-modulator of LMP1/NFκB activation, LMP1 up-regulates ABIN1 RNA levels (19). Inhibition pathway components also included the DUBs OTUD6B and USP8. OTUD6B is related to the OTU-domain family member, A20, the prototype NFκB down-modulator. Depletion of the NFκB negative-regulator TRIB3, a STK40-related RelA binding protein, also enhanced LMP1-mediated NFκB activation. Validated inhibitors included 11 Ub ligase subunits, six kinases, and seven phosphatase components. Negative NFκB regulators are among the most highly induced NFκB target genes, and TNFAIP2 depletion enhanced LMP1-mediated NFκB activation. TNFAIP2 is up-regulated by TNFα and also by mutation in lymphomas (48).

Discussion

These data identify and characterize multiple proteins important for LMP1-, TNFα–, and IL-1β–mediated NFκB regulation, thereby opening new opportunities to therapeutically regulate NFκB activation. Surprisingly, the LMP1 and IL-1β canonical NFκB activation pathways more closely resembled each other than the TNFα pathway. TRAF6 and its E2 enzyme UBC13, the only E2 used exclusively for K63 Ub-chain synthesis, were critical for LMP1 and IL-1β, but not for TNFα–induced NFκB activation. Together with the observation that IKK activation by IL-1β but not TNFα requires UBC13 and the Ub K63 residue (49), our results suggest that LMP1- and IL-1β–mediated NFκB activation are similarly K63-Ub dependent. In contrast, TNFα uses the UBC5 E2 and cIAP E3 Ub ligases to polyubiquitinate RIPK1 (49). Newly identified factors important for LMP1- and IL-1β–, but not TNFα–mediated NFκB activation, may activate TRAF6 or may be downstream targets of TRAF6 K63 Ub. Alternatively, they may be selectively important for LMP1 and IL-1R1 post-translational modification, trafficking, or stability. Proteins important for LMP1 and IL-1β NFκB activation are likely mediators of TLR-induced NFκB pathways, which are also TRAF6-dependent.

How different NFκB stimuli cause NFκB transcription factor occupancy of DNA binding sites is not well understood. Indeed, RelA DNA binding patterns differ in response to latent EBV infection versus TNFα stimulation, as judged by ChipSeq analyses (50). Multiple LMP1 activation pathway components were nuclear proteins that were differentially important for NFκB activation by TNFα or IL-1β (Fig. 4). Indeed, thirteen screen hits are zinc-finger proteins with predicted nuclear localization, of which two were important for LMP1 only, five for LMP1 and IL-1β, and six for LMP1, TNFα, and IL-1β; most are likely to be important downstream of IKK activation.

The human genome encodes 55 ZC3H-type zinc-finger proteins, at least five of which are important NFκB negative regulators. ZC3H proteins have multiple inhibitory roles in destabilization of RNAs that encode NFκB activation-pathway proteins (51). Consistent with this, ZC3H12a knockout causes lethal immune hyperactivation in mice (38), and ZC3H12b depletion increased LMP1-mediated NFκB induction. In contrast, ZC3H13 and ZC3H18 were instead important for NFκB activation. These nuclear phosphoproteins have putative RNA-binding zinc-finger motifs, but do not contain RNase or other recognizable domains. Interestingly, ZC3H-family proteins can also increase target mRNA abundance (52). ZC3H13 and ZC3H18 may therefore stabilize RNAs that encode NFκB activation pathway components, or may destabilize inhibition-pathway component mRNAs.

NFκB activation by oncogenic mutations are frequently observed in diffuse large B-cell lymphoma, Hodgkin lymphoma, multiple myeloma, and melanoma (53, 54). The genetic basis for elevated NFκB can be somatic mutations in NFκB activation- or inhibition-pathway components (53). Our NFκB pathway data enable broader searches for tumor-associated somatic or inherited mutations that drive malignant cell growth. Somatic mutations of the LMP1 activation-pathway proteins TRAF2 and TAK1 have been identified in lymphoma (55), of ZC3H13 in gastric and colon carcinoma (56), and of ZC3H18 in melanoma (57).

Genome-wide siRNA screens enable unbiased identification of proteins important for biological processes. We used 293 cells because they have low basal and highly inducible NFκB activity, are largely unaffected by NFκB blockade, and have been frequently used for NFκB-pathway research. These results enable focused testing of activation pathway and down-modulator protein roles in LMP1, TNFα, and IL-1β NFκB activation in other therapeutically relevant cell types. Although cell type differences are evident in NFκB pathway components, proteins identified in these studies are expressed across many cell types. Our data provide unique insights, approaches, and tools for investigating NFκB regulation, and are a useful resource for target-focused inhibitor discovery.

Experimental Methods

Primary and validation siRNA screens were performed with the Dharmacon siArray library. siRNAs were transfected into 293 cells at a 50 nM final concentration, using the Dharmafect lipid #1 (Thermo Fisher) in a 384-well format. GFP and DsRed values were quantitated by high-throughput FACS. Please refer to *SI Appendix* for detailed explanations of experimental methods. Luciferase values represent IκBα–*Photinus* luciferase/Renilla luciferase ratios in siRNA treated LMP1 induced cells, normalized to ratios in uninduced cells. Values in siRNA nontargeting control treated cells were defined as 100%. siRNAs used in figure preparation are presented in Dataset S7. Please see *SI Appendix* for full details.

ACKNOWLEDGMENTS. We thank E. Davis (MD Anderson) for IκBα–*Photinus* and Renilla luciferase vectors and technical assistance; T. Yasui (Osaka University) for Tet-On DsRed; D. Hill and M. Vidal (Harvard Medical School) for split YFP expression vectors; W. Khan (University of Miami) for a GST-IκBα expression vector; D. Portal for help with figure preparation; D. Boehm for helpful discussions and technical assistance, and G. Hoffman, A. Brass, J. Smith, C. Shamu, D. Wrobel, S. Rudnicki, S. Johnston, M. Padi, E. Bennett, M. Sowa, and J. W. Harper for helpful discussions. B.E.G. is a fellow of the

Leukemia and Lymphoma Society and the Karin Grunebaum Cancer Foundation. This research was supported by National Cancer Institute Grants K08CA140780 (to B.E.G.), R01CA085180 (to E.K.), R01CA47006 (to E.K.), and 5T32CA009031 (to N.S.), by National Human Genome Research Institute Grant HGS5P50HG004233 (to E.K. and J.Q.), by National Institute of Allergy and Infectious Diseases Grants U54AI057159 to the New England Regional Center of Excellence in Biodefense and Emerging Infectious Diseases and R01AI062773 (to R.J.X.), by National Institute of Diabetes, Digestive, and Kidney Diseases Grants R01043351 and R0183756 (to R.J.X.), and by National Center for Research Resources Harvard Catalyst Grant UL1RR025758 (to B.E.G.).

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