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# Oncogenic activity of BIRC2 and BIRC3 mutants independent of nuclear factor-κB-activating potential

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**Key words**

BIRC2, BIRC3, nuclear factor-κB, oncogene, NIK, resequencing

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**BIRC2 and BIRC3 are closely related members of the inhibitor of apoptosis (IAP) family of proteins and play pivotal roles in regulation of nuclear factor-κB (NF-κB) signaling and apoptosis. Copy number loss for and somatic mutation of *BIRC2* and *BIRC3* have been frequently detected in lymphoid malignancies, with such genetic alterations being thought to contribute to carcinogenesis through activation of the noncanonical NF-κB signaling pathway. Here we show that *BIRC2* and *BIRC3* mutations are also present in a wide range of epithelial tumors and that most such nonsense or frameshift mutations confer direct transforming potential. This oncogenic function of BIRC2/3 mutants is largely independent of their ability to activate NF-κB signaling. Rather, all of the transforming mutants lack an intact RING finger domain, with loss of ubiquitin ligase activity being essential for transformation irrespective of NF-κB regulation. The serine-threonine kinase NIK was found to be an important, but not exclusive, mediator of BIRC2/3-driven carcinogenesis, although this function was independent of NF-κB activation. Our data thus suggest that, in addition to the BIRC2/3–NIK–NF-κB signaling pathway, BIRC2/3–NIK signaling targets effectors other than NF-κB and thereby contributes directly to carcinogenesis. Identification of these effectors may provide a basis for the development of targeted agents for the treatment of lymphoid malignancies and other cancers with *BIRC2/3* alterations.**

Members of the inhibitor of apoptosis (IAP) family of proteins share a baculovirus IAP repeat (BIR) domain and play pivotal roles in the regulation of nuclear factor (NF)-κB signaling and apoptosis.[1,2] In response to activation of tumor necrosis factor receptors, for instance, BIRC2 (also known as cIAP1) and BIRC3 (also known as cIAP2), two closely related members of the IAP family, catalyze the ubiquitylation of RIPK1 in a manner dependent on their RING finger domains. This event triggers activation of the canonical NF-κB signaling pathway, which includes the phosphorylation of IKKγ and the consequent activation of RELA and p50 (a processed form of NFKB1). On the other hand, BIRC2/3 also ubiquitylate the serine-threonine kinase NIK (NF-κB-inducing kinase) and thereby prevent NIK-mediated activation of RELB and p52 (a processed form of NFKB2) in the noncanonical NF-κB pathway. BIRC2/3 thus have both stimulatory and inhibitory functions in the regulation of NF-κB signaling, with these functions being dependent on cell context.

Various somatic alterations of *BIRC2* and *BIRC3* genes have been identified in lymphoid malignancies. Gastric mucosa-associated lymphoid tissue (MALT) lymphoma may disappear in response to eradication of *Helicobacter pylori*, but it is unlikely to do so if the lymphoma cells have acquired the *BIRC3-MALT1* fusion-type oncogene. The transforming activity of BIRC3-MALT1 is thought to result from its marked ability to activate NF-κB signaling.[3]

In contrast, *BIRC2/3* are frequently inactivated by copy number loss or by nonsense or frameshift mutations in multiple myeloma.[4,5] Somatic mutations of *BIRC3* have also been detected in splenic marginal zone lymphoma[6] and mantle cell lymphoma.[7] In these instances, *BIRC2/3* mutations are loss-of-function, and are thought to contribute to carcinogenesis through activation of the noncanonical NF-κB signaling pathway.

To identify transforming genes in non-small cell lung cancer (NSCLC), we have now analyzed exome DNA and selected cDNAs derived from the lung squamous cell carcinoma cell line H1703 with the use of a next-generation sequencer (NGS). We detected a nonsense mutation in *BIRC3* and found that this mutation confers direct transforming potential on the protein product. Somatic nonsense or insertion/deletion (indel) mutations that result in loss of the RING finger domain of BIRC3 were found to be present in a wide range of epithelial tumors and were also shown to be oncogenic. Unexpectedly, the transforming potential of BIRC3 mutants was found not to be directly related to their ability to activate NF-κB signaling. Likewise, most oncogenic *BIRC2* mutations found in cancer did not result in the activation of NF-κB. Our observations indicate that transforming mutants of BIRC2/3 exert their effects, at least in part, through an NF-κB-independent pathway that likely depends on the ubiquitylation of target molecules including NIK.

## Materials and Methods

**Cell lines.** Human embryonic kidney 293T (HEK293T), human NSCLC H1703, and 3T3 mouse fibroblast cell lines were obtained from American Type Culture Collection (Manassas, VA, USA) and were maintained in Dulbecco's modified Eagle's medium-F12 (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and 2 mM L-glutamine (both from Invitrogen).

**NGS analyses.** Exon fragments were isolated from genomic DNA of H1703 cells with the use of a SureSelect Human All Exon kit (Agilent Technologies, Santa Clara, CA, USA) and were subjected to NGS analysis with the HiSeq2500 platform with the paired-end option (Illumina, San Diego, CA, USA). From the large datasets, we selected only sequence reads with a Q value of ≥20 at each base, and further extracted unique reads that were subsequently mapped to the reference human genome sequence (hg19) with the use of the Bowtie 2 algorithm (http://bowtie-bio.sourceforge.net/bowtie2/index.shtml).

Custom RNA probes of 120 bases were designed to capture cDNAs for 5463 human protein-coding genes (Table S1) and were synthesized by Agilent Technologies. The cDNAs were captured from H1703 cells as described previously,[8] and similarly sequenced with HiSeq2500. NGS reads were mapped to the RefSeq database (http://www.ncbi.nlm.nih.gov/refseq) with the Bowtie 2 algorithm.

Mismatches were discarded if: (i) a given read contained ≥3 independent mismatches; (ii) they were already present in the "1000 genomes" database (http://www.1000genomes.org) or in the normal human genome variations of our in-house database; or (iii) they were supported by only one strand of the genome. Gene mutations were annotated with SnpEff (http://snpeff.sourceforge.net).

**Functional analyses.** Methods for functional analyses are described in Data S1.

## Results

**Transforming potential of BIRC3(E358*).** We performed whole-exome sequencing for the lung squamous cell carcinoma cell line H1703 with an NGS at a mean coverage of 105× in order to identify transforming genes in NSCLC. We also performed cDNA-capture sequencing[8] for 5463 cancer-related genes, with the cDNAs of these genes being enriched with the use of custom-made capture probes. Nonsynonymous nucleotide substitutions detected in these analyses (with thresholds for total coverage of ≥30× and for the mutation ratio of ≥20%) included 56 alterations in 53 independent genes (Table S2). Most ($n$ = 44) of the 56 mutations thus identified were found to be already reported in the public databases of cancer genome alterations including COSMIC version 61 (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic), Cancer Cell Encyclopedia (CCE, http://www.broadinstitute.org/ccle/home), International Cancer Genome Consortium (ICGC, https://icgc.org), and The Cancer Genome Atlas (TCGA, http://cancergenome.nih.gov).

Among the nonsynonymous mutations in H1703, we found a nonsense mutation in *BIRC3*, mutations of which had been frequently reported in lymphoid malignancies.[3–6] We thus tested if the identified BIRC3(E358*) has a direct contribution to carcinogenesis of NSCLC. Interestingly, as shown in Figure 1(a), BIRC3(E358*) clearly induced focus formation in 3T3 cells in culture, and also tumor formation in a nude mouse tumorigenicity assay, with the corresponding wild-type protein having no effects.

The E358* mutant of BIRC3 lacks the RING finger domain that confers ubiquitin ligase (E3) activity. We therefore tested whether loss of this enzymatic activity might contribute to malignant transformation. Histidine-574 in the RING finger domain is a key residue for ubiquitin ligase activity of BIRC3, with substitution of this amino acid abolishing E3 activity.[9,10] We thus generated the catalytic-null mutant BIRC3(H574A) and examined its transforming potential. As demonstrated in Figure 1(a), oncogenic activity of BIRC3(H574A) was found to be similar to that of BIRC3(E358*) both *in vitro* and *in vivo*. Expression level of the wild-type and the mutant forms of BIRC3 was examined by an immunoblot analysis (Suppl. Fig. S1). While the immunoblot band corresponding to BIRC3 or BIRC3(H574A) was broad compared to that to BIRC3 (E358*) probably owing to ubiquitination of the proteins,[11] a densitometric analysis of Figure S1 suggested that comparable amounts of the three proteins were expressed (1.23 arbitrary units for BIRC3, 1.25 for BIRC3(E358*) and 1.27 for BIRC3 (H574A)).

Given that BIRC3 is known to regulate the noncanonical NF-κB pathway, we next examined whether these BIRC3 mutants activate NF-κB. As shown in Figure 1(b), abundance of p52 and RELB was profoundly increased in the nucleus of 3T3 cells expressing BIRC3(E358*) or BIRC3(H574A), which suggests activation of the noncanonical NF-κB pathway. On the other hand, activation of the canonical NF-κB pathway was modest, demonstrated only by the BIRC3(H574A)–mediated increase in nuclear p50.

We further tested BIRC3-mediated NF-κB activation with a luciferase reporter assay. Figure 1(c) demonstrates that BIRC3 (E358*) was found to slightly upregulate the trans-activation activity of NF-κB in transfected HEK293T cells compared to the wild-type protein and that BIRC3(H574A) markedly induced an increase in NF-κB activity. These reporter activities were normalized by the expression level of the corresponding proteins (Suppl. Fig. S2).

To investigate the relation between the ability of BIRC3 to promote oncogenesis and its NF-κB-activating potential, we generated a series of truncation mutants of BIRC3(H574A) by independently deleting the BIR1, BIR2, BIR3, ubiquitin-associated (UBA) domains or the caspase recruitment domain (CARD). The BIR1 and UBA domains are required for homodimerization of and ubiquitin binding by BIRC3, respectively, and deletion of these domains attenuated the upregulation of NF-κB activity (Fig. 1d and Suppl. Fig. S3). We found that, in addition to the BIR1 and UBA domains, the BIR3 domain was essential for the induction of NF-κB activity in transfected cells. On the other hand, deletion of BIR2 or CARD domains enhanced the NF-κB activity.

Importantly, however, the transforming activity of the various BIRC3 mutants was not concordant with the ability to induce NF-κB activity (Fig. 1e). Deletion of the UBA domain, for instance, resulted in an increase in the transforming activity of BIRC3(H574A), suggesting that such activity is, at least in part, independent of NF-κB signaling.

**Nonsynonymous mutations of *BIRC3* in human cancer.** Several nonsynonymous mutations of *BIRC3* are reported in the COSMIC database, with most of these changes affecting the region of the encoded protein downstream of the BIR3 domain (Fig. 2a and Suppl. Table S3). It is of note that the BIRC3-MALT1 fusion oncoprotein lacks the entire RING finger domain of BIRC3. We found that all of the identified nonsense and frameshift mutations of BIRC3 confer oncogenic potential both *in vitro* (Fig. 2b) and *in vivo* (Suppl. Fig. S4). Given that

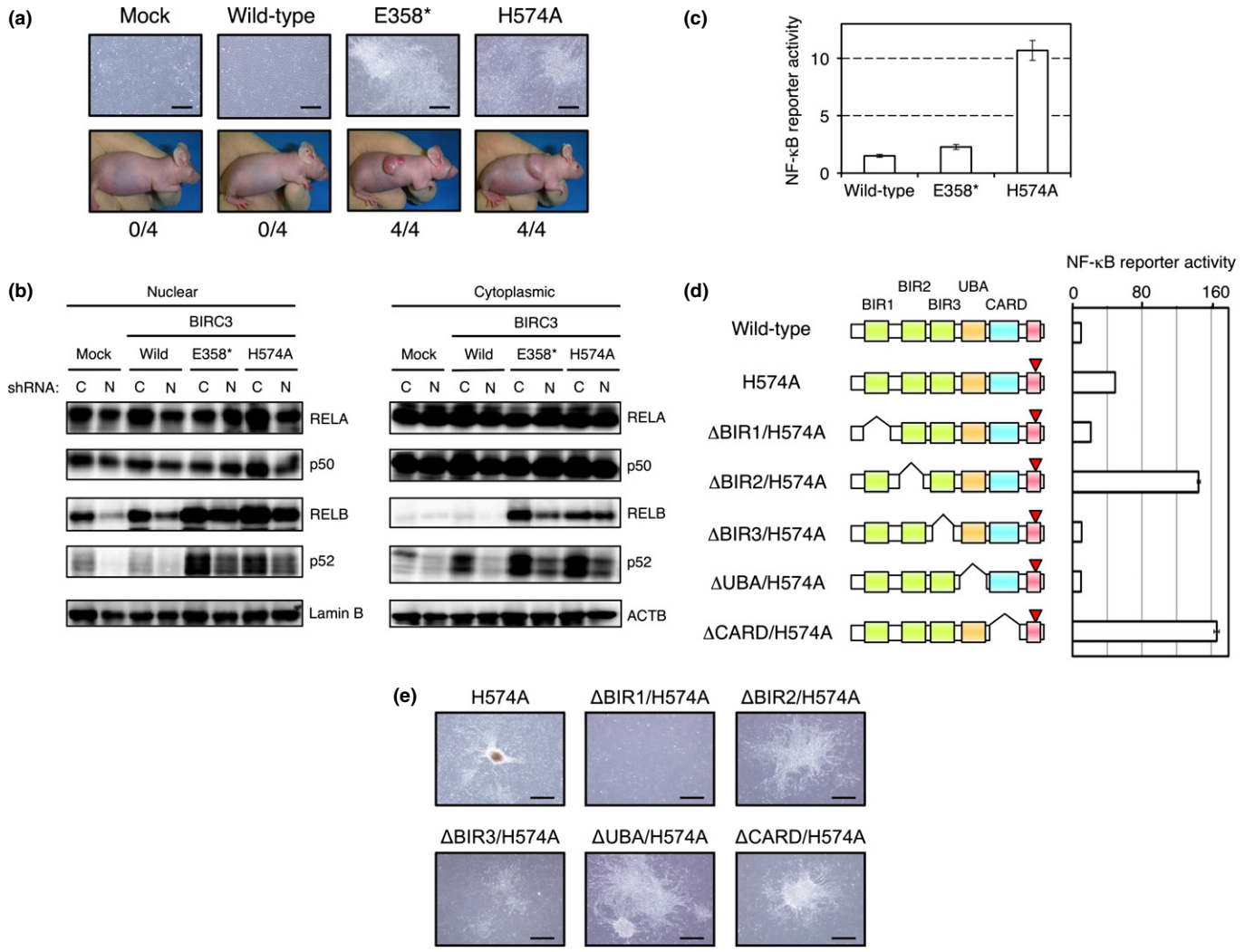


**Fig. 1.** Identification of BIRC3(E358*) as an oncoprotein. (a) A focus formation assay was performed with mouse 3T3 cells infected with retroviruses encoding either wild-type, E358* or H574A mutant forms of BIRC3 or with cells infected with the corresponding empty virus (Mock). The cells were examined by phase-contrast microscopy after culture for 2 weeks (upper panels). Scale bars, 0.5 mm. The same set of 3T3 cells was also injected subcutaneously into nu/nu mice, and the number of tumors at the injection site ($n = 4$) was determined after 28 days (lower panels). (b) Mouse 3T3 cells expressing control (C) or *Nik* (N) shRNA were infected with the empty retrovirus (Mock) or recombinant retrovirus encoding either wild-type, E358* or H574A mutant forms of BIRC3. Nuclear (left panel) or cytoplasmic (right) fractions of these cells were prepared and subjected to immunoblot analyses with antibodies to RELA, p50, RELB, p52, Lamin B or ACTB as indicated at the right. (c) Luciferase reporter activity was measured for HEK293T cells transfected with pMXS (Mock) or pMXS-based expression plasmids for wild-type or E358* or H574A mutant forms of BIRC3 as well as with a nuclear factor (NF)-κB reporter plasmid and the pGL4.70 plasmid for *Renilla* luciferase. Data represent firefly luciferase activity normalized by *Renilla* luciferase activity and the amounts of the corresponding proteins, and are shown as means ± SD of three independent experiments. (d) Schematic representations of the domain organization of BIRC3 and its truncation mutants (red arrowhead indicates the H574A mutation) are shown together with the NF-κB reporter activity for each construct measured as in (c). (e) Transforming activity of BIRC3(H574A) and its indicated truncation mutants was examined with a focus formation assay as in (a).

these mutants lack the RING finger domain, loss of ubiquitin ligase activity likely plays an essential role in their oncogenic activity. In contrast, all missense mutations failed to manifest transforming activity, with the exception that BIRC3(C319Y) showed a low transformation potential *in vitro* but did not generate tumors *in vivo*.

We also examined the various mutants for their ability to induce NF-κB activity (Fig. 2c). NF-κB reporter activities normalized by the expression level of the corresponding BIRC3 proteins (Suppl. Figs. S2 and S5) revealed that some of the transformation-inducing mutants, including E368* and V395 fs*6, did not activate NF-κB. The ability to activate NF-κB is thus not a prerequisite for oncogenic activity of BIRC3.

**Role of NIK in BIRC3-dependent transformation.** We hypothesized that substrates for ubiquitylation by BIRC3 are likely mediators of BIRC3-driven transformation, with NIK in particular being a promising mediator given that it is directly ubiquitylated by BIRC3[12] and that cells that harbor *BIRC3* mutations are dependent on NIK for survival.[13] We therefore examined whether NIK is required for BIRC3-dependent oncogenesis.

Among several small interfering RNAs (siRNAs) targeted to *Nik* mRNA (Suppl. Fig. S6), we chose siRNA #2 as the basis for construction of a short hairpin RNA (shRNA) for further experiments. Mouse 3T3 cells stably expressing the control or *Nik* shRNA were infected with retroviruses encoding wild-type or mutant forms of BIRC3 and then examined for malignant

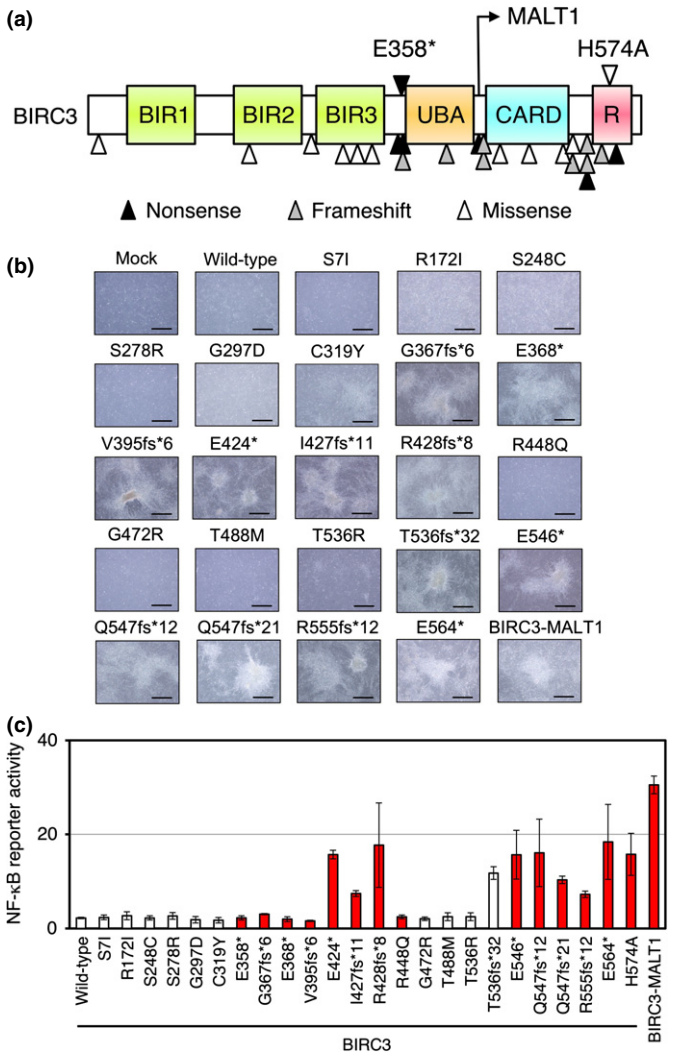


**Fig. 2.** Functional characterization of BIRC3 mutants found in human cancers. (a) Positions of various mutations identified in cancer in relation to the domain organization of the BIRC3 protein. Nonsense, frameshift, and missense mutations are indicated by black, gray, and white arrowheads, respectively. The fusion point to MALT1 and the H574A catalytic-null mutation are also shown. R, RING finger domain. (b) Focus formation assay performed with 3T3 cells expressing wild-type or the indicated mutant forms of BIRC3 as in Figure 1(a). Scale bars, 0.5 mm. (c) Nuclear factor (NF)-κB reporter activity measured in HEK293T cells expressing wild-type or the indicated mutant forms of BIRC3 as in Figure 1(b). BIRC3 mutants shown to possess transforming activity in vitro and in vivo are indicated in red. Data are means ± SD of three independent experiments.

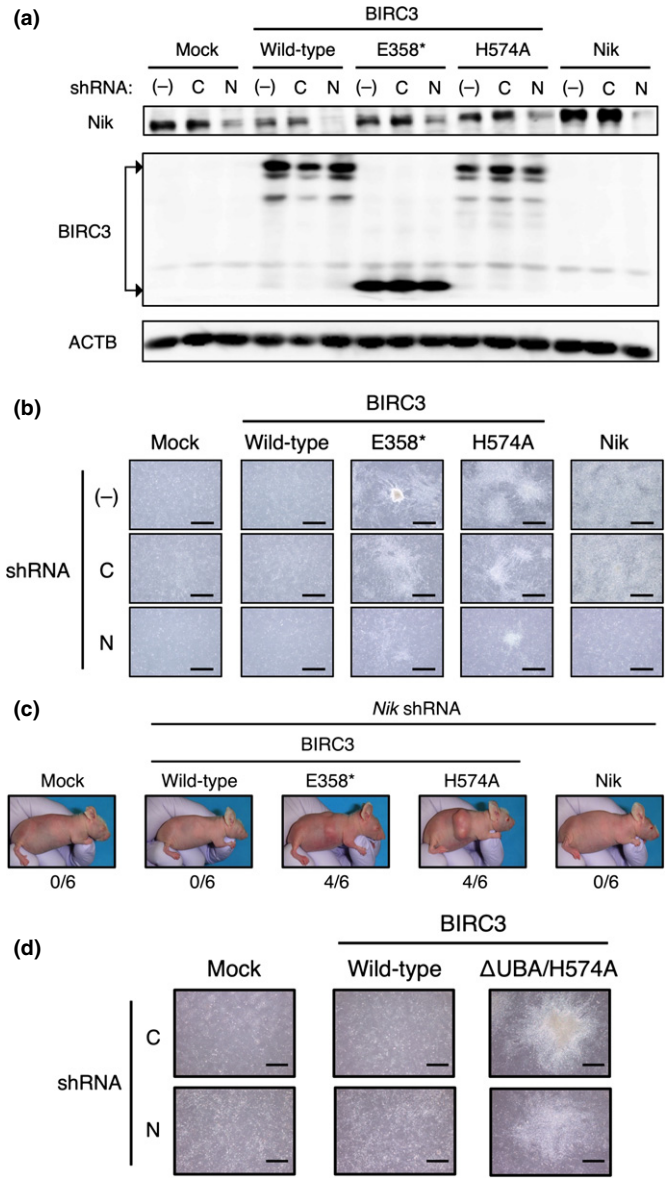


**Fig. 3.** Role of Nik in transformation driven by BIRC3 mutants. (a) Mouse 3T3 cells stably expressing control (C) or *Nik* (N) shRNAs, or those stably infected with the corresponding empty lentivirus (–), were infected with retroviruses encoding either wild-type or E358* or H574A mutant forms of human BIRC3 or mouse Nik (or with the corresponding empty retrovirus, Mock). The cells were then incubated for 3 h in the presence of the proteasome inhibitor MG132 (20 μM), lysed, and subjected to immunoblot analysis with antibodies to Nik, BIRC3 or ACTB as indicated at the left. (b) The same set of 3T3 cells as in (a) was subjected to a focus formation assay as in Figure 1(a). Scale bars, 0.5 mm. (c) Mouse 3T3 cells expressing *Nik* shRNA as well as the indicated forms of BIRC3 or wild-type Nik were assayed for tumorigenicity in nu/nu mice as in Figure 1(a), with the exception that the number of tumors at the injection sites ($n = 6$) was determined after 42 days. (d) Mouse 3T3 cells expressing control (–) or *Nik* (N) shRNA were infected with the empty retrovirus (Mock) or recombinant retrovirus expressing the wild-type or ΔUBA/H574A mutant of BIRC3, and were subjected to the focus formation assay. Scale bars, 0.5 mm.

potential. While significant expression of *Nik* mRNA was observed in 3T3 cells (data not shown), immunoblot analysis barely detected Nik protein, due to rapid ubiquitination and degradation. We thus pre-treated 3T3 cells with a proteasome inhibitor, MG132, for 3 hr before the detection of the Nik protein.

Immunoblot analysis confirmed that the abundance of Nik was markedly reduced in all cells expressing *Nik* shRNA (Fig. 3a). The transforming activity of BIRC3(E358*) or BIRC3(H574A) was substantially, but not completely, attenuated in cells expressing *Nik* shRNA in both *in vitro* and *in vivo* (Fig. 3b,c). Further, *Nik* knockdown suppressed BIRC3 mutant-mediated increase of p52 in the nucleus (Fig. 1b). The residual transforming ability of the two BIRC3 mutants did not appear to be due to residual Nik protein, given that forced expression of an shRNA-sensitive form of mouse *Nik* in the cells expressing the *Nik* shRNA did not induce malignant transformation *in vitro* or *in vivo*. Expression of wild-type *Nik*

in the absence of the *Nik* shRNA, on the other hand, induced marked transformation of 3T3 cells (Fig. 3b).

Further, to confirm the presence of Nik-dependent but NF-κB-independent mechanism in the BIRC3-mediated transformation pathway, we examined if *Nik* knockdown attenuates focus formation of 3T3 by BIRC3(ΔUBA/H574A) that does not have an ability to activate NF-κB (Fig. 1d). As shown in Figure 3(d), BIRC3(ΔUBA/H574A)-driven transformation of 3T3 cells was significantly weakened by knockdown of the *Nik* messages.

**Transforming potential of *BIRC2*.** Given that the protein structure of BIRC2 is almost identical to that of BIRC3, we searched the COSMIC, CCE, ICGC, and TCGA databases for nonsynonymous mutations of BIRC2. Most of the identified mutations were nonsense or frameshift and were localized in the carboxyl-terminal half of the protein (Fig. 4a and Suppl. Table S3). With the exception of a frameshift mutation at Lys-19, all of the mutations rendered BIRC2 oncogenic *in vitro* (Fig. 4b and Suppl. Fig. S7). As in the case of BIRC3, a catalytic-null mutant of BIRC2, BIRC2(H588A), also manifested transforming potential.

We also examined the various BIRC2 mutants for the ability to induce NF-κB activation. Whereas a frameshift mutation at Glu-440 and the artificial mutation at His-588 increased the ability of BIRC2 to activate NF-κB, such effect was marginal for some mutants and others even suppressed NF-κB (Fig. 4c), indicative of an NF-κB-independent transformation mechanism for BIRC2 as for BIRC3.

Finally, wild-type BIRC2 or BIRC2(H588A) was introduced into 3T3 cells stably expressing *Nik* shRNA in order to examine whether Nik is required for BIRC2-dependent oncogenesis. Similar to the case for BIRC3, the transforming ability of BIRC2(H588A) was attenuated but not abolished in cells depleted of Nik (Fig. 4d). *Nik* shRNA also suppressed the BIRC2(H588A)-mediated increase of p52 in the nucleus (Fig. 4e).

## Discussion

We have here revealed a direct transforming potential of BIRC2 and BIRC3 mutants that is, at least in part, unrelated to the ability of these mutants to regulate NF-κB signaling. Rather, loss of the RING finger domain and the consequent inability of the mutants to ubiquitylate substrates likely play a central role in their induction of oncogenesis.

Given that knockdown of *NIK* mRNA suppresses the growth of mantle cell lymphoma cell lines in which the noncanonical NF-κB pathway is activated,[13] and that NIK depletion markedly attenuated BIRC2/3 mutant-mediated 3T3 cell transformation, NIK is likely a major downstream effector of BIRC2/3 mutants in the induction of oncogenesis. However, the mechanism by which loss of BIRC2/3 enzymatic activity and consequent overexpression of NIK trigger transformation remains unclear. NIK-mediated activation of the noncanonical NF-κB pathway (phosphorylation of IKKα and subsequent limited proteolysis of NFKB2) is not essential for the transformation

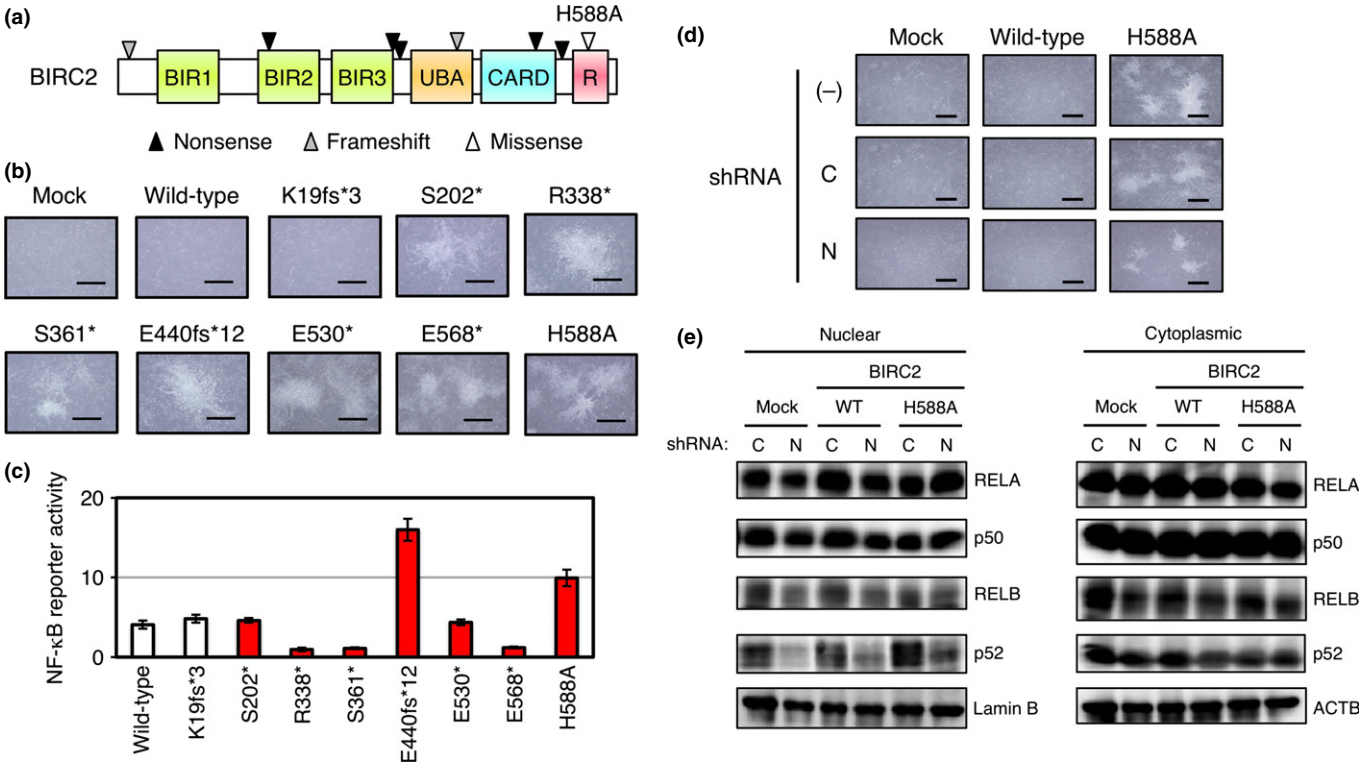


**Fig. 4.** Oncogenic activity of BIRC2 mutants. (a) Domain organization of BIRC2 showing the positions of cancer-associated mutations and the H588A catalytic-null mutation. (b) Focus formation assay performed with 3T3 cells expressing wild-type or the indicated mutant forms of BIRC2 as in Figure 1(a). Scale bars, 0.5 mm. (c) Nuclear factor (NF)-κB reporter activity measured in HEK293T cells expressing wild-type or the indicated mutant forms of BIRC2 as in Figure 1(b). BIRC2 mutants shown to possess transforming activity *in vitro* are indicated in red. Data are means ± SD of three independent experiments. (d) Mouse 3T3 cells stably expressing control (C) or *Nik* (N) shRNAs, or those stably infected with the corresponding empty lentivirus (–), were infected with retroviruses encoding wild-type or H588A mutant forms of BIRC2 (or with the corresponding empty retrovirus, Mock). The cells were then assayed for focus formation. Scale bars, 0.5 mm. (e) Nuclear or cytoplasmic fractions of the cells in (d) were subjected to immunoblot analyses with antibodies to RELA, p50, RELB, p52, Lamin B or ACTB as in Figure 1(b).

mechanism, given that some BIRC2/3 mutants with full transforming potential failed to activate NF-κB.

Our observations are consistent with a recent study of *Nik*$^{-/-}$ mice.[14] These mice develop eosinophilia that resembles human hypereosinophilia. Unexpectedly, however, such eosinophilia does not appear to result from aberrant NIK-IKKα signaling. Mice expressing an IKKα mutant that cannot be phosphorylated by NIK were thus defective in NFKB2 processing but were found to have normal numbers of eosinophils. These observations suggest that NIK also functions in an intracellular signaling pathway independent of IKKα-p52.

Moreover, BIRC2/3-driven carcinogenesis may be mediated by downstream players other than NIK, given that NIK depletion did not completely abrogate BIRC2/3-induced malignant transformation. NIK and other targets that are ubiquitylated by BIRC2/3 (and subsequently degraded by the proteasome) in normal cells therefore likely become oncoproteins after ubiquitylation is suppressed. Whereas little information is currently available on such targets, they may associate with the BIR1 or BIR3 domain, given that deletion of either of these domains attenuated the transforming ability of BIRC3(H574A).

Nonsense or frameshift mutations of *BIRC2/3* are frequently found in B cell malignancies, but they have also been detected in a wide range of epithelial tumors (Table S3). Given that most such BIRC2/3 mutants lack the RING finger domain and therefore possess direct transforming ability, identification of the molecules that mediate BIRC2/3-driven carcinogenesis may provide a basis for the development of new targeted drugs for the treatment of such cancers.

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## Disclosure Statement

The authors declare no conflict of interest.

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## Supporting Information

Additional supporting information may be found in the online version of this article:

**Data S1.** Supplementary methods.

**Fig. S1.** Expression level of BIRC3 mutants in 3T3.

**Fig. S2.** Expression level of BIRC3 mutants in HEK293T.

**Fig. S3.** Expression level of truncation mutants of BIRC3.

**Fig. S4.** Tumorigenicity assay for BIRC3 mutants associated with human cancers.

**Fig. S5.** Expression level of non-transforming mutants of BIRC3 in HEK293T.

**Fig. S6.** Knockdown efficiency of *Nik* siRNAs.

**Fig. S7.** Expression level of BIRC2 mutants.

**Table S1.** Genes interrogated in custom cDNA-capturing system.

**Table S2.** Nonsynonymous nucleotide substitutions in H1703.

**Table S3.** Known nonsynonymous mutations within *BIRC2* and *BIRC3*.