LncRNA NBR2 engages a metabolic checkpoint by regulating AMPK under energy stress

Xiaowen Liu1,8, Zhen-Dong Xiao1,8, Leng Han2, Jiexin Zhang3, Szu-Wei Lee4,5, Wenqi Wang1, Hyemin Lee1, Li Zhuang1, Junjie Chen1,5, Hui-Kuan Lin4,5,6,7, Jing Wang1, Han Liang3 and Boyi Gan1,4,5,9

Long non-coding RNAs (lncRNAs) have emerged as critical regulators in various cellular processes. However, the potential involvement of lncRNAs in kinase signalling remains largely unknown. AMP-activated protein kinase (AMPK) acts as a critical sensor of cellular energy status. Here we show that the lncRNA NBR2 (neighbour of BRCA1 gene 2) is induced by the LKB1–AMPK pathway under energy stress. On energy stress, NBR2 in turn interacts with AMPK and promotes AMPK kinase activity, thus forming a feed-forward loop to potentiate AMPK activation during energy stress. Depletion of NBR2 attenuates energy-stress-induced AMPK activation, resulting in unchecked cell cycling, altered apoptosis/autophagy response, and increased tumour development in vivo. NBR2 is downregulated and its low expression correlates with poor clinical outcomes in some human cancers. Together, the results of our study uncover a mechanism coupling lncRNAs with metabolic stress response, and provides a broad framework to understand further the regulation of kinase signalling by lncRNAs.

Mammalian genomes encode more than 10,000 long non-coding RNAs (lncRNAs), RNA molecules that are longer than 200 nucleotides and do not seem to encode proteins1,2. Although lncRNAs were traditionally viewed as the products that are generated from the background noise of transcription and thus exert little fitness advantage to the cells, it has become increasingly clear that these lncRNAs play important biological functions, and their dysregulation has been connected to various human diseases, including cancer3–6.

Most current studies focus on lncRNA function in the nucleus, partly because most of the best-understood lncRNAs, such as XIST (ref. 7), HOTAIR (ref. 8), HOTTIP (ref. 9), are all chromatin-associated lncRNAs, which are mainly localized in the nucleus. These studies have illustrated a diverse range of functions of lncRNAs in the regulation of chromatin status, transcription and RNA processing, among others10. Many lncRNAs have also been identified in the cytosol11. In fact, it has been suggested that most lncRNAs probably spend most of their lifetime in the cytoplasm1. However, the exact functions of cytoplasmic localized lncRNAs, particularly their potential functions in the regulation of kinase signalling in the cytoplasm, remain poorly understood. In addition, although lncRNAs have been shown to regulate diverse biological processes, the role of lncRNAs in mediating a metabolic checkpoint remains largely unexplored.

The AMP-activated protein kinase (AMPK) serves as a critical sensor of cellular energy status and is activated under energy stress conditions with an increased cellular AMP/ATP ratio12. AMP binding to AMPK and subsequent AMPK phosphorylation at Thr172 by the upstream kinase LKB1 leads to AMPK activation13–15. Activated AMPK then phosphorylates a number of downstream targets to inactivate ATP-consuming anabolic processes and to activate ATP-generating catabolic processes16. Thus, AMPK mainly functions as a metabolic checkpoint to restore energy balance in response to energy stress. One major anabolic process inhibited by AMPK in response to energy stress is mammalian target of rapamycin complex 1 (mTORC1)-mediated protein synthesis and cell growth17. In response to energy stress, AMPK inactivates mTORC1 and represses protein synthesis through AMPK phosphorylation of Raptor, a component of mTORC1, and the TSC1–TSC2 complex, a negative regulator of mTORC1 (refs 18,19). AMPK also functions to promote autophagy and cell survival under energy stress through its phosphorylation of autophagy regulators, such asULK1 (refs 20,21). As anabolic

1Department of Experimental Radiation Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA.
2Department of Biochemistry and Molecular Biology, The University of Texas Health Science Center at Houston McGovern Medical School, Houston, Texas 77030, USA.
3Department of Bioinformatics and Computational Biology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA.
4Department of Molecular and Cellular Oncology, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, Texas 77030, USA.
5Program of Genes and Development, and Program of Cancer Biology, The University of Texas Graduate School of Biomedical Sciences, 1515 Holcombe Boulevard, Houston, Texas 77030, USA.
6Department of Cancer Biology, Wake Forest School of Medicine, Winston-Salem, North Carolina 27157, USA.
7Graduate Institute of Basic Medical Science, China Medical University, Taichung 404, Taiwan. *These authors contributed equally to this work.
8Correspondence should be addressed to B.G. (e-mail: bgan@mdanderson.org)

Received 24 February 2015; accepted 10 February 2016; published online 21 March 2016; DOI: 10.1038/ncb3328
Figure 1 Energy stress induces NBR2 expression through the LKB1–AMPK pathway. (a,b) Various cell lines were cultured in 0 or 25 mM glucose-containing medium (a), or 0 or 5 mM 2DG-containing medium (b) for 12–24 h, and then subjected to real-time PCR analysis to measure NBR2 expression (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (c,d) HeLa or A549 cells stably expressing empty vector (EV) or LKB1 expression vectors were cultured in 25 or 0 mM glucose-containing medium, and then subjected to real-time PCR (c) (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test) and western blotting analyses (d). (e) MDA-MB-231 cells treated with 100 nM A769662 were subjected to real-time PCR analysis to measure NBR2 expression (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (f) MDA-MB-231 cells were treated with 20µM compound C in 25 or 0 mM glucose-containing medium for 24 h, and then subjected to real-time PCR analysis to measure NBR2 expression (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (g) MDA-MB-231 cells transfected with AMPKα or control (Ctrl) siRNA were cultured in 25 or 0 mM glucose-containing medium for 24 h, and then subjected to real-time PCR analysis to measure NBR2 expression (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). Source data for a–c,e,g can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplementary Fig. 8.

processes, such as protein and lipid synthesis, often exert pro-growth effects in tumour development, it is well documented that AMPK activation serves to inhibit tumour development in many cancers. Consistent with this, both the upstream kinase LKB1 and downstream effectors of AMPK, such as TSC1 and TSC2, are bona fide tumour suppressors and are mutated in hamartoma tumour syndromes and various sporadic cancers. Although the biological functions of AMPK and its downstream effectors
involved in cancer development have been extensively studied\textsuperscript{22,26}, the regulatory mechanisms of AMPK activation by energy stress remain incompletely understood. In particular, it remains completely unknown whether any lncRNA is involved in the AMPK-mediated metabolic checkpoint.

In this study, we identify neighbour of BRCA1 gene 2 (\textit{NBR2}) as an energy-stress-induced lncRNA and show that \textit{NBR2} interacts with AMPK and potentiates AMPK activation under energy stress. Consistent with the tumour suppression function of AMPK, \textit{NBR2} deficiency promotes unchecked cell cycling under energy stress and enhances tumour development \textit{in vivo}, and \textit{NBR2} is downregulated in human cancers. Our study thus reveals a previously unappreciated regulatory mechanism by lncRNAs to regulate kinase function and to mediate cellular energy responses.

**Figure 2** \textit{NBR2} regulates AMPK–mTORC1 signalling under energy stress. (a) Bar graph showing \textit{NBR2}-shRNA-mediated knockdown efficiency by real-time PCR analysis in 786-O and MDA-MB-231 cells (mean \pm s.d., \textit{n}=3 biologically independent extracts, two-tailed paired Student’s \textit{t}-test). (b,c) 786-O or MDA-MB-231 cells infected with either control shRNA or \textit{NBR2} shRNA were cultured in medium with different concentrations of glucose for 24 h. Cell lysates were then analysed by western blotting. (d) 786-O or MDA-MB-231 cells infected with either control shRNA or \textit{NBR2} shRNA were cultured in 0 or 5 mM 2DG-containing medium for 12 (for MDA-MB-231 cells) or 16 (for 786-O cells) h. Cell lysates were then analysed by western blotting. (e) MDA-MB-231 cells infected with either control shRNA or \textit{NBR2} shRNA were cultured in 0 or 100 \mu M A769662-containing medium for 12 h. Cell lysates were then analysed by western blotting. Source data for (a) can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
RESULTS

Energy stress induces NBR2 expression through the LKB1–AMPK pathway

To identify energy-stress-induced IncRNAs, we conducted an RNA sequencing experiment in 786-O cells that had been cultured in glucose-containing or glucose-free medium. Subsequent computational analysis identified NBR2 as one of the long intergenic non-coding RNAs (lincRNAs) induced by glucose starvation. The NBR2 gene encodes different splicing isoforms ranging from 1 to 2 kilobases (Supplementary Fig. 1). It has been shown that NBR2 is expressed in most of the tissues examined. However, the NBR2 gene does not seem to encode a protein, and its potential function remains unknown.

Real-time PCR revealed that glucose starvation induced NBR2 expression in different cancer cell lines, except HeLa and A549 cells, which are LKB1 deficient (Fig. 1a). Treatment with the glucose analogue 2-deoxy-glucose (2DG), another energy stress inducer that inhibits hexokinase and blocks glycolysis, yielded similar results (Fig. 1b). Importantly, re-expression of LKB1 inhibited hexokinase and blocks glycolysis, yielded similar results (Fig. 1c) with AMPK (ref. 15) treatment or siRNA-mediated NBR2 expression (Fig. 1e), whereas AMPK inactivation by compound C (an AMPK inhibitor) treatment or siRNA-mediated AMPKα knockdown significantly attenuated glucose starvation-induced NBR2 expression (Fig. 1f,g and Supplementary Fig. 2). Together, our results revealed that energy stress induces NBR2 expression at least partly through the LKB1–AMPK pathway.

NBR2 regulates AMPK–mTORC1 signalling under energy stress

To study the potential function of NBR2 in mediating energy stress response, we generated 786-O cells (a kidney cancer cell line) and MDA-MB-231 cells (a breast cancer cell line) with stable knockdown of NBR2 (Fig. 2a). We then analysed whether knockdown of NBR2 affected any biochemical signalling surrogate induced by energy stress, including AMPK activation. As shown in Fig. 2b, glucose starvation potently induced phosphorylation of AMPK, or the AMPK substrates acetyl-CoA carboxylase (ACC) and Raptor18,22. Notably, NBR2 knockdown significantly attenuated glucose-starvation-induced phosphorylation of AMPK, ACC and Raptor. Accordingly, S6 and S6K dephosphorylation induced by glucose deprivation was significantly compromised in NBR2 knockdown cells compared with control short hairpin RNA (shRNA)-infected cells (Fig. 2c). Finally, NBR2 knockdown also attenuated 2DG- or A769662-treatment-induced AMPK activation and mTORC1 inactivation (Fig. 2d,e). Our results thus revealed that NBR2 depletion attenuates energy stress-induced AMPK activation and mTORC1 inactivation, and suggested a feed-forward mechanism on NBR2–AMPK regulation, in which AMPK initially promotes NBR2 expression in response to energy stress and NBR2 in turn regulates AMPK activation under energy stress (see Discussion).

NBR2 regulates cell proliferation, apoptosis and autophagy in response to energy stress

AMPK functions as a critical metabolic checkpoint; defective AMPK signalling leads to increased cell proliferation yet decreased autophagy under conditions of energy stress, leading to apoptosis12,20. The aforementioned data prompted us to examine the impact of NBR2 deficiency on cell proliferation, apoptosis and autophagy in response to energy stress. Glucose starvation markedly decreased S phase entry as measured by BrdU incorporation, and knockdown of NBR2 significantly attenuated the reduction of S phase entry on glucose starvation (Fig. 3a–c). Thus, similar to cells with defective AMPK signalling18, NBR2-deficient cells continue cycling under energy stress. Although NBR2 depletion did not affect apoptosis under normal culture conditions, NBR2 deficiency induced more apoptosis under glucose starvation, as evidenced by both Annexin V staining (Fig. 3d,e) and cleaved caspase-3 western blotting (Fig. 3f). In response to energy stress, AMPK activates autophagy, a cellular adaptive response to promote cell survival under stress conditions20,21. Accordingly, glucose-starvation-induced GFP–LC3 puncta formation, p62 degradation and ULK1 phosphorylation were significantly compromised in NBR2-deficient cells (Fig. 3g,h and Supplementary Fig. 3a,b), suggesting that energy-stress-induced autophagy was defective in NBR2-deficient cells. Despite enhanced apoptosis, the number in NBR2-deficient cells increased under glucose-deprived conditions because of the increase in cycling in NBR2-deficient cells (Fig. 3i,j and Supplementary Fig. 3c,d). Collectively, our results showed that NBR2 deficiency leads to enhanced cell cycling yet decreased autophagy and increased apoptosis under energy stress, which is in line with the phenotypes from cells with defective AMPK signalling, including AMPK–, LKB1–, TSC1– and TSC2-deficient cells or cells reconstituted with a Raptor mutant that is non-phosphorylatable by AMPK (refs 15,18,19,29,30).

NBR2 inhibits tumour development and is downregulated in human cancers

Given the important functions of AMPK in the regulation of human cancers22, we next examined the potential roles of NBR2 in tumour development. NBR2 deficiency led to increased anchorage-independent growth, one of the hallmarks of cell transformation, with a more prominent effect under glucose-starvation conditions (Fig. 4a,b). In vivo experiments using the xenograft model showed that NBR2 deficiency increased tumour development (Fig. 4c). Further analyses of the tumour samples by western blotting confirmed downregulation of AMPK and upregulation of mTORC1 signalling in NBR2-deficient tumours (Fig. 4d).

Consistent with the experimental results from breast and renal cancer cell lines, a survey of the RNA-seq data across different cancer types from the TCGA (The Cancer Genome Atlas) data sets revealed downregulation of NBR2 expression in breast (BRCA) and renal (KIRC) cancer samples compared with paired normal tissue samples (Fig. 4e,f). Kaplan–Meier analysis showed that breast cancer patients with NBR2-low tumours had significantly worse overall survival than those with NBR2-high tumours (Fig. 4g). Together, our data showed that NBR2 deficiency promotes tumour development, and NBR2 is downregulated in human breast and renal cancers, suggesting that NBR2 may function as a tumour suppressor in these cancers.

Energy stress induces NBR2 interaction with AMPK

The aforementioned biological data prompted us to further study how NBR2 regulates AMPK function. Real-time PCR analyses of
Figure 3 NBR2 regulates cell proliferation, apoptosis and autophagy in response to energy stress. (a) Bar graph showing the percentages of S phase (BrdU positive) cells in control-shRNA- or NBR2-shRNA-infected MDA-MB-231 cells that were cultured in 25 or 0 mM glucose-containing medium for 24 h (mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student's t-test). (b,c) Bar graph showing the -glucose/+glucose ratio of S phase percentages in control-shRNA- or NBR2-shRNA-infected 786-O cells (b) or MDA-MB-231 cells (c) (mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student's t-test). (d-f) Control-shRNA- or NBR2-shRNA-infected 786-O cells or MDA-MB-231 cells were cultured in medium with different concentrations of glucose for 24 h, and then subjected to Annexin V/PI staining followed by FACS analysis to measure the percentages of Annexin V-positive/PI-negative cells (d for 786-O cells, e for MDA-MB-231 cells; mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student's t-test), or to western blotting analysis to measure caspase-3 cleavage (f). (g,h) Bar graph showing the percentages of cells with LC3–GFP punctate localization in control-shRNA- or NBR2-shRNA-infected 786-O cells (g) or MDA-MB-231 cells (h), which were transfected with GFP–LC3 and then cultured in 25 or 0 mM glucose-containing medium for 12 (for MDA-MB-231 cells) or 18 (for 786-O cells) h (mean ± s.d., n=5 fields per group, each field was assessed from an independent experiment, two-tailed paired Student's t-test). Source data for a–e,i,j can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
fractionated nuclear and cytoplasmic RNA revealed that NBR2 localized in both the nucleus and the cytoplasm (Fig. 5a). As expected, AMPKα showed predominant localization in the cytoplasm (Fig. 5b). AMPK exists as a heterotrimeric complex that consists of a catalytic α subunit and two regulatory β and γ subunits. We thus examined whether NBR2 can interact with any of the subunits of AMPK by RNA-pulldown assay using in vitro-synthesized biotinylated NBR2. Such analysis revealed that NBR2 interacted with overexpressed AMPKα under glucose-starvation conditions, with minimal binding with overexpressed β or γ subunit (Fig. 5c). The RNA-pulldown assay also revealed that glucose starvation significantly increased the interaction of NBR2 with endogenous AMPKα (Fig. 5d). As AMPKα, β and γ subunits form a very stable complex at the endogenous level, we also observed a glucose-starvation-induced binding between NBR2 and endogenous AMPKβ and γ subunits (Fig. 5d), probably mediated by NBR2 interaction with the endogenous AMPKα subunit.

In vitro binding assay using purified AMPKα and in vitro-synthesized biotinylated NBR2 confirmed the direct binding between NBR2 and AMPKα (Fig. 5e). There exist at least three splicing isoforms of the NBR2 gene (named as NBR2 no. 1, no. 2 and no. 3; see Supplementary Fig. 1). In the RNA-pulldown experiments described above, we used the NBR2 no. 1 splicing isoform. The RNA-pulldown experiments showed that the NBR2 no. 2 and no. 3 splicing isoforms also interacted with AMPKα on glucose starvation (Fig. 5f). Finally,
RNA immunoprecipitation assay (using primers that can detect all three NBR2 splicing isoforms) revealed an enrichment of NBR2 in the precipitates of AMPKα compared with the IgG control, and glucose starvation substantially increased the enrichment of NBR2 in AMPKα precipitates (note that glucose starvation resulted in a much higher fold increase of the NBR2 level in AMPKα precipitates compared with the NBR2 input level) (Fig. 5g).

In the experiment to map the region(s) of AMPKα that mediates AMPK interaction with NBR2, we showed that the kinase-domain-containing amino-terminal region, but not the carboxy-terminal
region of AMPKα, interacted with NBR2 (Fig. 5h). Mutation of Thr172 to alanine in AMPKα did not affect AMPKα interaction with NBR2 (Fig. 5h), indicating that AMPK phosphorylation at Thr172 is not required for AMPK–NBR2 interaction. Together, our data revealed that glucose starvation not only induces NBR2 expression, but also enhances NBR2 interaction with AMPK, which is possibly mediated by NBR2 interaction with the kinase domain of AMPKα.

**NBR2 promotes AMPK kinase activity**

Next we studied the underlying mechanisms by which NBR2 regulates AMPK function. To this end, we first examined whether overexpression of NBR2 exerts any biological effect in cells. Our experiments revealed that overexpression of any of the three splicing isoforms resulted in AMPK activation, mTORC1 inactivation (Fig. 6a,b), and decreased cell proliferation without affecting apoptosis under normal culture conditions (Fig. 6c). All three splicing isoforms of NBR2 share the same first two exons located at the 5’ end of NBR2 with distinctive exons located towards the 3’ end (Supplementary Fig. 1). Our data thus indicate that the common exons in all NBR2 splicing isoforms might be important in mediating NBR2 interaction with AMPK. Consistent with this, our binding mapping experiments revealed that the first exon shared by all three NBR2 splicing isoforms is both required and sufficient to mediate NBR2 interaction with AMPKα (Fig. 6d). Furthermore, overexpression of the T1 fragment of NBR2 no. 1, which lacks the first exon (with 159 base pairs (bp) out of 1,045 bp full-length NBR2 no. 1) and thus is incapable of interacting with AMPK, did not affect AMPK and mTORC1 activation status or cell proliferation, whereas in the parallel experiments, overexpression of full-length NBR2 no. 1 exerted the expected effects on AMPK signalling (Fig. 6e,f). It seems that overexpression of the first exon alone (T4 fragment of NBR2 no. 1) was not sufficient to activate AMPK (Supplementary Fig. 4a), suggesting that other regions in NBR2 may be also important for NBR2 function in the regulation of AMPK. Together, our results showed that deletion of the first exon of NBR2 abolishes its interaction with AMPK and regulation of AMPK activation, suggesting that NBR2 regulation of AMPK activation and downstream cellular processes is probably mediated through NBR2 interaction with AMPK.

As LKB1 functions as the main upstream kinase of AMPK in response to energy stress13–15, we examined whether NBR2 regulates LKB1 interaction with AMPK. Our results showed that NBR2 overexpression or knockdown did not affect AMPK–LKB1 interaction under either basal or glucose-starvation conditions (Supplementary Fig. 4b,c). In addition, we found that overexpression of NBR2 in LKB1-deficient HeLa cells could still promote AMPK activation, and co-expression of NBR2 and LKB1 in HeLa cells led to a synergic increase of AMPK activation (Supplementary Fig. 4d). Together, our data suggest that NBR2 does not regulate AMPK–LKB1 interaction and it is likely that NBR2 operates in parallel to LKB1 to regulate AMPK activation.

Our data showing that NBR2 interacts with the kinase domain of AMPKα (Fig. 5h) prompted the hypothesis that NBR2 may directly regulate the kinase activity of the AMPK complex. Our data showed that bacterially purified GST–ACC (amino acids 1–130) could be readily phosphorylated by the AMPK complex precipitated from cell lysates of HEK293T cells co-transfected with AMPKα/β/γ constructs (Fig. 6g). Whereas *in vitro*-synthesized NBR2 alone did not lead to ACC phosphorylation, the addition of NBR2 (but not the T1 fragment of NBR2, the AMPK non-binding mutant) to the AMPK complex significantly increased ACC phosphorylation by AMPK (Fig. 6g). The *in vitro* kinase assay using purified AMPKα/β/γ complex and SAMS peptide as the AMPK substrate further confirmed that NBR2 promoted AMPK *in vitro* kinase activity (Fig. 6h). Together, our data suggest that NBR2 functions to promote AMPK kinase activity possibly through its interaction with the AMPK kinase domain.

**The functional effects of NBR2 are partially mediated by AMPK**

We next sought to determine the extent to which the functional effects of NBR2 are mediated by NBR2 regulation of AMPK activation. We first examined whether overexpression of NBR2 still exerted its functional effects in AMPKα knockout cells. Such analyses revealed that, although overexpression of NBR2 increased ACC phosphorylation, decreased S6 phosphorylation, and suppressed cell proliferation in control (Ctrl)-siRNA-transfected cells, such effects were attenuated in AMPKα knockout (AMPK siRNA) cells (Fig. 7a,b). As a complementary approach, we also examined whether restoration of constitutively active (CA) AMPK (amino acids 1–312 of AMPKα1) would rescue any of the defects observed in NBR2-deficient cells. Our data revealed that overexpression of AMPK CA in NBR2 knockout cells restored ACC or S6 phosphorylation under glucose-starvation conditions as expected (Fig. 7c), and correspondingly, significantly rescued cell proliferation, apoptosis, and anchorage-independent growth under glucose-starvation conditions in NBR2-deficient cells (Fig. 7d–g). Importantly, restoration of AMPK CA in the NBR2-deficient background significantly attenuated the enhanced xenograft tumour development caused by NBR2 deficiency (Fig. 7h). Taken together, our data strongly suggested that the functional effects of NBR2 are at least partially dependent on AMPK.

**DISCUSSION**

AMPK exists as a heterotrimeric complex comprising of a catalytic α subunit and two regulatory β and γ subunits, in which the γ subunit directly binds to AMP in response to energy stress31. It has been proposed that AMP activates AMPK through at least three mechanisms: AMP binding to AMPKα causes allosteric activation of AMPK, and leads to conformational change of the AMPK complex that promotes Thr172 phosphorylation in the AMPKα subunit by LKB1 and inhibits Thr172 dephosphorylation by protein phosphatases31. Our study reveals that lincRNA NBR2 regulation of AMPK represents another important regulatory mechanism to control AMPK activation in response to energy stress. Here we propose a feed-forward model on NBR2–AMPK regulation. Specifically, energy-stress-induced initial AMPK activation does not require NBR2. Activated AMPK then upregulates NBR2 expression in response to energy stress. NBR2 in turn interacts with AMPK and promotes AMPK kinase activity under energy stress, forming a feed-forward loop to potentiate AMPK activation during chronic energy stress conditions (Supplementary Fig. 5a). NBR2 deficiency leads to AMPK inactivation during long periods of energy stress, which promotes mTORC1 activation, cell proliferation and tumour development (Supplementary Fig. 5b). As transcription regulation in general takes a
Figure 6 NBR2 promotes AMPK kinase activity. (a, b) Protein lysates were prepared from HEK293T (a) or UMRC2 cells (b) with overexpression of EV or NBR2 expression vectors, and analysed by western blotting. (c) UMRC2 cells stably expressing EV or NBR2 expression vectors were cultured in 25 mM glucose-containing medium for different days as indicated, and then subjected to cell proliferation analysis (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (d) Top: Schematic diagram showing different truncation mutants of NBR2 no. 1 and the summary of their binding capabilities to AMPK. Bottom: In vitro-synthesized biotinylated sense (S), antisense (AS), or different truncation (T) mutants of NBR2 no. 1 were incubated with protein lysates from 786-O cells that had been cultured in glucose-free medium for 24 h. Precipitation reactions were conducted using streptavidin beads and then subjected to western blotting. (e) Protein lysates were prepared from HEK293T or UMRC2 cells with overexpression of EV, NBR2 no. 1 full length (FL), or T1 mutant expression vectors, and analysed by western blotting. (f) UMRC2 cells stably expressing EV, NBR2 no. 1 FL, or T1 mutant expression vectors were cultured in 25 mM glucose-containing medium for different numbers of days as indicated, and then subjected to cell proliferation analysis (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (g) AMPK complex precipitated from HEK293T cells was subjected to the kinase assay in the presence of ATP, in vitro-synthesized RNAs and GST–ACC 1–130 amino acid fusion proteins as indicated. The kinase activity of AMPK was measured by phosphorylation of ACC at the Ser79 site. (h) In vitro-purified active human AMPK complex was subjected to in vitro kinase assays in the presence of ATP, SAMS peptide and in vitro-synthesized biotinylated sense (S)/antisense (AS)/T1 mutant (T1) NBR2 no. 1 or several chemical compounds (compound C, A769662, AMP) as indicated (see Methods for details). The kinase activity was measured by the luminescence with a plate-reading illuminometer (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). Source data for c, f, h can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
Figure 7 The functional effects of NBR2 are partially mediated by AMPK. (a,b) UMRC2 cells stably expressing EV or NBR2 expression vectors were transfected with AMPK siRNA (AMPK siRNA1 or siRNA2) or control (Ctrl) siRNA. Protein lysates were prepared and analysed by western blotting (a), or cells were cultured in 25 mM glucose-containing medium for different numbers of days as indicated, and then subjected to cell proliferation analysis (b) (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). (c-g) MDA-MB-231 cells with stable expression (Ctrl) shRNA or NBR2 shRNA were infected with empty vector (EV) or constitutively active AMPK (AMPK CA). These cells were cultured in 25 or 0 mM glucose-containing medium for 24 h, and protein lysates were prepared and analysed by western blotting (c); the cells were cultured in 25 mM glucose-containing medium for different numbers of days as indicated, and then subjected to crystal violet staining to measure cell number (d) (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test); the cells were cultured in 25 or 0 mM glucose-containing medium for 24 h, and then subjected to Annexin V/PI staining followed by FACS analysis to measure the percentages of Annexin V-positive/PI-negative cells (e) (mean ± s. d., n = 5 fields per group, each field was assessed from an independent experiment, two-tailed paired Student’s t-test); the cells were cultured in 25 or 0 mM glucose-containing medium for different numbers of days as indicated, and then subjected to crystal violet staining to measure cell number (d) (mean ± s.d., n = 3 biologically independent extracts, two-tailed paired Student’s t-test). Bar graph showing the mean colony numbers from the soft agar assay (g) (mean ± s.d., n = 5 fields per group, each field was assessed from an independent experiment, two-tailed paired Student’s t-test). Source data for b,d,e can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplementary Fig. 8.
longer time than allosteric regulation and phosphorylation events, we reason that cells may have evolved this lincRNA-involved regulatory mechanism to maintain AMPK activation during long periods of energy stress and to help cells adapt better to chronic stress conditions. In support of this model, our time course experiments revealed that NBR2 deficiency compromised AMPK activation at later, but not earlier, time points on glucose starvation (Supplementary Fig. 6a). (Note that all of the energy stress experiments shown in our studies used 12 h or longer treatment time points.) This mirrors well with the kinetics of NBR2 expression induction on glucose starvation (Supplementary Fig. 6b). As glucose starvation also significantly promotes NBR2 binding to AMPK (Fig. 5), this presumably further amplifies the effect of NBR2 to promote AMPK activation.

The NBR2 gene was originally identified as a gene that is located near to the breast-cancer-associated gene BRCA1. Both genes lie head to head with each other on human chromosome 17, and the physical distance between the transcription start sites of the two genes is only 218 bp (Supplementary Fig. 1)27. Given the frequent mutation/deletion rates of BRCA1 in human breast and ovarian cancers and the close proximity of the NBR2 gene to the BRCA1 gene, it was initially postulated that NBR2 should be co-deleted/mutated with BRCA1 in certain cancers (for example, see ref. 32), and NBR2 may also play a role in tumour suppression. However, later it became clear that NBR2 does not seem to encode a protein, and it was proposed that NBR2 simply is a ‘junk gene’33. Since then, its potential function in tumour biology has remained unknown. In this study, we identified NBR2 as a lincRNA induced by energy stress, and showed that NBR2 indeed functions to inhibit tumour development, at least in part through its regulation of AMPK activation. It is of note that NBR2 overexpression in AMPK−/− cells can still exert moderate cell proliferation suppressive effect (Fig. 7b), suggesting that NBR2 may have other AMPK-independent function(s) to regulate cell proliferation. Identification and characterization of other NBR2-binding proteins or RNAs will further clarify its function.

The most popular model proposed for lncRNA function probably is the one whereby lncRNAs regulate gene expression, either in cis or in trans, through recruiting other chromatin-modification complexes or transcription factors to specific loci34,35. This raises the possibility that NBR2 may regulate the transcription of the BRCA1 gene, which resides right next to the NBR2 gene. However, our data showed that BRCA1 expression was not affected by either glucose starvation or NBR2 knockdown (Supplementary Fig. 7). We should mention that, although initially it was proposed that lincRNAs mainly function to regulate neighbouring gene transcription, other studies have shown that many lincRNAs do not exert such a function36. Whether NBR2 regulates any other gene transcription awaits further investigation.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Supplementary Information is available in the online version of the paper.

ACKNOWLEDGEMENTS

We thank all members of the Gan laboratory for their advice and technical assistance. This research has been supported by grants from MD Anderson Cancer Center, US Department of Defense (TS093049), Cancer Prevention & Research Institute of Texas (RP130020), National Institutes of Health (CA181196 and CA190370), Ellison Medical Foundation (AG-NS-0973-13), and Gabrielle’s Angel Foundation for Cancer Research (to B.G.). B.G. is a Kimmel Scholar and Ellison Medical Foundation New Scholar. H.Liang is supported by the National Institutes of Health (CA143883, CA175486); the R. Lee Clark Fellow Award from The Jeanne F. Shelby Scholarship Fund; a grant from the Cancer Prevention and Research Institute of Texas (RP140462); and the Mary K. Chapman Foundation and the Lorraine Dell Program in Bioinformatics for Personalization of Cancer Medicine. L.H. is supported by Cancer Prevention & Research Institute of Texas (RR150085). H.-K.L. is supported by the National Institutes of Health (CA182424 and CA190383). B.G., J.C., J.W. and H.Liang are members of the MD Anderson Cancer Center, and are supported by the National Institutes of Health Core Grant CA016672.

AUTHOR CONTRIBUTIONS

Z.-D.X. initiated the project and identified NBR2 as an energy-stress-induced lincRNA. X.L. performed most of the experiments with assistance from Z.-D.X., H.Lee, and L.Z.J.Z. and J.W. analysed RNA-Seq data set. L.H. and H.Liang conducted computational analysis on NBR2 expression and status in human cancers. W.W., J.C., S.-W.L. and H.-K.L. provided reagents. B.G. supervised the study. X.L., Z.-D.X. and B.G. designed the experiments and wrote the manuscript. All authors commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

Published online at http://dx.doi.org/10.1038/ncomms3328

Reprints and permissions information is available online at www.nature.com/reprints

1. Uiltisky, I. & Bartel, D. P. lincRNAs: genomics, evolution, and mechanisms. Cell 154, 26–46 (2013).
2. EP Consortium, An integrated encyclopedia of DNA elements in the human genome. Nature 489, 57–74 (2012).
3. Batista, P. J. & Chang, H. Y. Long noncoding RNAs: cellular address codes in development and disease. Cell 152, 1298–1307 (2013).
4. Gupta, R. A. et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464, 1071–1076 (2010).
5. Huarte, M. et al. A large intergenic noncoding RNA induced by p53 mediates global gene repression in the p53 response. Cell 142, 403–419 (2010).
6. Preinser, J. R. et al. Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression. Nat. Biotechnol. 29, 742–749 (2011).
7. Engertz, J. M. et al. The Xist lncRNA exploits three-dimensional genome architecture to spread across the X chromosome. Science 341, 1237973 (2013).
8. Rinn, J. L. et al. Functional demarcation of active and silent chromatin domains in human HGX loci by noncoding RNAs. Cell 129, 1311–1323 (2007).
9. Wang, K. C. et al. A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression. Nature 472, 120–124 (2011).
10. Guttmann, M. & Rinn, J. L. Modular regulatory principles of large non-coding RNAs. Nature 482, 339–346 (2012).
11. van Heesch, S. et al. Extensive localization of long noncoding RNAs to the cytosol and mono- and polyribosomal complexes. Genome Biol. 15, R6 (2014).
12. Hardie, D. G., Ross, F. A. & Hawley, S. A. AMPK: a nutrient and energy sensor that maintains energy homeostasis. Nat. Rev. Mol. Cell Biol. 13, 251–262 (2012).
13. Hawley, S. A. et al. Complexes between the LKB1 tumor suppressor, STRAD α/β and MO25 α/β are upstream kinases in the AMP-activated protein kinase cascade. J. Biol. Chem. 278, 28 (2003).
14. Wood, A. et al. LKB1 is the upstream kinase in the AMP-activated protein kinase cascade. Curr. Biol. 13, 2004–2008 (2003).
15. Shaw, R. J. et al. The tumour suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc. Natl Acad. Sci. USA 101, 3329–3335 (2004).
16. Hardie, D. G., Schaffer, B. E. & Brunet, A. AMPK: an energy-sensing pathway with multiple inputs and outputs. Trends Cell Biol. 26, 190–201 (2015).
17. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. Cell 149, 274–293 (2012).
18. Gwinn, D. M. et al. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol. Cell 30, 214–226 (2008).
19. Inoki, K., Zhu, T. & Guan, K. L. TSC2 mediates cellular energy response to control cell growth and survival. Cell 115, 577–590 (2003).
20. Egan, D. F. et al. Phosphorylation of ULK1 (hATG1) by AMP-activated protein kinase connects energy sensing to mitophagy. Science 331, 456–461 (2011).
21. Kim, J., Kundu, M., Voellet, B. & Guan, K. L. AMPK regulates autophagy through direct phosphorylation of Ulk1. Nat. Cell Biol. 13, 132–141 (2011).
22. Shackelford, D. B. & Shaw, R. J. The LKB1-AMPK pathway: metabolism and growth control in tumour suppression. Nat. Rev. Cancer 9, 563–575 (2009).
23. Huang, J. & Manning, B. D. The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem. J. 412, 179–190 (2008).
24. Hezel, A. F. & Bardeesy, N. LKB1; linking cell structure and tumor suppression. Oncogene 27, 6908–6919 (2008).
25. Alessi, D. R., Sakamoto, K. & Bayascas, J. R. LKB1-dependent signaling pathways. Annu. Rev. Biochem. 75, 137–163 (2006).
26. Faubert, B. et al. AMPK is a negative regulator of the Warburg effect and suppresses tumor growth in vivo. Cell Metab. 17, 6908–6919 (2008).
27. Xu, C. F. et al. Isolation and characterisation of the NBR2 gene which lies head to head with the human BRCA1 gene. Hum. Mol. Genet. 6, 1057–1062 (1997).
28. Sim, A. T. & Hardie, D. G. The low activity of acetyl-CoA carboxylase in basal and glucagon-stimulated hepatocytes is due to phosphorylation by the AMP-activated protein kinase and not cyclic AMP-dependent protein kinase. FEBS Lett. 233, 294–298 (1988).
29. Bungard, D. et al. Signaling kinase AMPK activates stress-promoted transcription via histone H2B phosphorylation. Science 329, 1201–1205 (2010).
30. Conradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A. & Guan, K. L. Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev. 18, 1533–1538 (2004).
31. Hardie, D. G. AMPK-sensing energy while talking to other signaling pathways. Cell Metab. 20, 939–952 (2014).
32. Gad, S. et al. Characterisation of a 161 kb deletion extending from the NBR1 to the BRCA1 genes in a French breast-ovarian cancer family. Hum. Mutat. 21, 654 (2003).
33. Jin, H. et al. Structural evolution of the BRCA1 genomic region in primates. Genomics 84, 1071–1082 (2004).
34. Pandey, R. R. et al. Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. Mol. Cell 32, 232–246 (2008).
35. Feng, J. et al. The Evf-2 noncoding RNA is transcribed from the Dlx-5/6 ultraconserved region and functions as a Dlx-2 transcriptional coactivator. Genes Dev. 20, 1470–1484 (2006).
METHODS

Cell culture studies. Human kidney cancer cell lines, human breast cancer cell lines, human prostate cancer cell lines, human embryonic kidney 293 cells used in this study were mostly obtained from American Type Culture Collection (ATCC). All of the plasmids were purchased from Origene (SR303721, SR303722). All three splicing isoforms of NBR2 through LR Gateway Technology (Invitrogen). Active human AMPK were obtained from Thermo Fisher Scientific (MGC human AMPK1/2, F6) mouse monoclonal antibody (Cell Signaling Technology, D63G4, 1:5,000 dilution), Flag-AMPK1 and Flag-AMPK2 entry plasmids were obtained from the Human ORFeome V5.1 library. The entry clones were subsequently recombined into gateway-compatible destination vectors with Flag tag through LR Gateway Technology (Invitrogen). DNA corresponding to amino acids 1-312 of AMPK1 was cloned into entry vector, and was subsequently recombined into gateway-compatible destination vectors with V5 tag through LR Gateway Technology (Invitrogen). Active human AMPKalpha2 and active human AMPKalpha1+AMPKbeta1+AMPKgamma1 protein were purchased from Abcam (ab79863, ab126916). 2-Deoxy-D-glucose and compound C were purchased from Sigma (D6134, P5499). A-769662 was purchased from LC laboratories (A-1803). Quantitative real-time PCR and RNA immunoprecipitation (RIP) assay. Total RNA was extracted from cells using RNAeasy (Qiagen) and first-strand cDNA was prepared with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ABI). Real-time PCR was performed using the Quantitect SYBR Green PCR kit (Qiagen) or TaqMan Universal PCR Master Mix (ABI), and was run on the Stratagene MX3000P. For quantification of gene expression, the 2^-ΔΔCt method was used. GAPDH expression was used for normalization. RIP assay was performed with the Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore). Briefly, cells were lysed in RIP lysis buffer. Then the lysates were immunoprecipitated with antibody or IgG along with protein magnetic beads. After proteinase K digestion, the RNAs pulled down with proteins were purified by phenol chloroform extraction and precipitated in ethanol. The RNAs were then resuspended in RNase-free water and cDNA was synthesized and subjected to real-time PCR to detect NBR2 or GAPDH (internal control) transcripts. The RNA level was normalized with input (10%).

NATURE CELL BIOLOGY DOI: 10.1038/ncl3328 © 2016 Macmillan Publishers Limited. All rights reserved.
Xenograft model. All animal experiments with female athymic Nude–Foxn1nu mice (6-week-old) were performed in accordance with a protocol approved by the Institutional Animal Care and Use Committee of MD Anderson Cancer Center, which is in full compliance with policies of the Institutional Animal Core and Use Committee (IACUC). Animals arriving in our facility were randomly put into cages with five mice each. Tumour volume measurement was initiated at two weeks after injection (defined as starting time point: week 0). Tumour progression was then monitored by bi-dimensional tumour measurements every five days using a caliper until the endpoint. Mice were euthanized at the endpoint and the tumours were excised for further experiments. The tumour volume was calculated according to the equation

\[ V = \frac{4}{3} \pi \cdot \left( \frac{a \cdot b}{2} \right)^{3/2} \]

where \( V \) is the tumour volume at week \( n \), and \( a \) and \( b \) are the tumour length and width, respectively. For calculating the tumour volume at week \( n \) expressed as relative tumour volume (RTV) and calculated according to the following formula:

\[ RTV = \frac{V_n}{V_0} \]

where \( V_n \) is tumour volume at week \( n \) and \( V_0 \) is the tumour volume at week 0. The investigators were not blinded to allocation during experiments and outcome assessment.

RNA-seq and computational analysis. RNA-seq was performed at the Sequencing and Non-Coding RNA Program at the MD Anderson Cancer Center using Applied Biosystems SOLiD Next Generation Sequencing platform. LifeScope v2.3.1 was used to align the reads to the genome, generate raw counts corresponding to each known gene (total 23,080 genes, including 4,325 non-coding genes), and calculate the RPMK (reads per kilobase per million) values. We considered only non-coding genes that expressed at relatively high levels (RPKM > 0.5-fold changes) and showed >2- or <0.5-fold changes between control and treatment cells. This identified a list of 17 upregulated and 39 downregulated non-coding RNAs.

Kaplan–Meier survival analysis of cancer patients. We used data sets of 4,142 breast tumours that had previously been profiled by Affymetrix microarray analysis (www.kmplot.com)\(^{28}\); NBR2 expression (probe set ID: 207631_at) was divided by the median into high versus low expression. Survival analysis by Kaplan–Meier and Cox proportional hazard analysis was performed.

TCGA data analysis. We downloaded the level-3 gene expression data for NBR2 from the TCGA pan-cancer project Synapse (Synapse ID: syn300013) for breast (BRCA) and kidney cancer (KIRC). We used paired Student’s \( t \)-test to detect the overall survival difference between patient groups.

Accession numbers. RNA-seq data sets (786-O cells with or without glucose suspension at 1.0 mmol/L) were independently repeated 35 times. For survival analysis, the expression of NBR2 was treated as a binary variable and divided into `high' and `low' level. Kaplan–Meier survival curves were compared using the Gehan-Breslow test with \( P < 0.05 \), \( P < 0.01 \), and \( P < 0.001 \), as indicated in the individual figures. For animal studies, five mice per group is the standard sample size for tumour xenograft experiments, and no statistical method was used to predetermine sample size. None of the samples/animals was excluded from the experiment, and the animals were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

Western blotting, representative images are shown. Each of these experiments was independently repeated 3–5 times. For survival analysis, the expression of NBR2 was treated as a binary variable and divided into 'high' and 'low' level. Kaplan–Meier survival curves were compared using the Gehan–Breslow test with GraphPad Prism (GraphPad Software). The experiments were not randomized. The investigators were not blinded to allocation during experiments and outcome assessment.

36. Lee, S. W. et al. Skp2-dependent ubiquitination and activation of LKB1 is essential for cancer cell survival under energy stress. Mol. Cell 57, 1022–1033 (2015).
37. Lin, A. et al. The FOXO-BNP3 axis exerts a unique regulation of mTORC1 and cell survival under energy stress. Oncogene 33, 3183–3194 (2014).
38. Gan, B. et al. Lkb1 regulates quiescence and metabolic homeostasis of haematopoietic stem cells. Nature 468, 701–704 (2010).
39. Gan, B. et al. mTORC1-dependent and -independent regulation of stem cell renewal, differentiation, and mobilization. Proc. Natl Acad. Sci. USA 105, 19384–19389 (2008).
40. Gan, B. et al. FoxOs enforce a progression checkpoint to constrain mTORC1-activated renal tumorigenesis. Cancer Cell 18, 472–484 (2010).
41. Lin, A. et al. FoxO transcription factors promote AKT Ser473 phosphorylation and renal tumor growth in response to pharmacological inhibition of the PI3K-AKT pathway. Cancer Res. 74, 1682–1693 (2014).
42. Gyoryf, B., Surowia, P., Burdacz, J. & Lancerzyk, A. Online survival analysis software to assess the prognostic value of biomarkers using transcriptomic data in non-small-cell lung cancer. PLoS ONE 8, e82241 (2013).
43. Gyoryf, B. et al. An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1,809 patients. Breast Cancer Res. Treat. 123, 725–731 (2010).
**Supplemental Figure 1** The schematic diagram of the genomic region of human *BRCA1* and *NBR2* genes with different splicing isoforms. Arrows represent the direction of transcription.
Supplemental Figure 2 AMPK inactivation by compound C or AMPKα siRNA treatment. (a) MDA-MB-231 cells were treated with 20 µM Compound C in 25 or 0 mM glucose-containing medium for 24 hours, and then subjected to Western blotting analysis to measure AMPK activation. (b) MDA-MB-231 cells transfected with AMPKα or control (Ctrl) siRNA were cultured in 25 or 0 mM glucose-containing medium for 24 hours, and then subjected to Western blotting analysis to measure AMPK activation. Unprocessed original scans of blots are shown in Supplemental Fig. 8.
Supplemental Figure 3  NBR2 knockdown affects autophagy and cell proliferation in response to energy stress. (a) The effect of NBR2 deficiency on GFP-LC3 puncta formation. 786-O cells infected with either control shRNA or NBR2 shRNA were transfected with GFP-LC3 plasmid, and then cultured in 25 or 0 mM glucose-containing medium for 18 hours. GFP-LC3 punctate foci were then detected using fluorescence microscopy. (Scale bars, 20 µm)  (b) The effect of NBR2 deficiency on ULK1 phosphorylation and p62 degradation in response to glucose starvation. MDA-MB-231 cells infected with either control shRNA or NBR2 shRNA were cultured in 25 or 0 mM glucose-containing medium for 12h. Cell lysates were then analyzed by Western blotting.  (c, d) Cells infected with either control shRNA or NBR2 shRNA were cultured in 1mM glucose-containing medium for different days as indicated, and then subjected to cell proliferation analysis (Mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student’s t-test). Source data for c, d can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplemental Fig. 8.
Supplemental Figure 4 Mechanistic studies of NBR2 regulation of AMPK.
(a) Protein lysates were prepared from HEK293T cells transfected with empty vector (EV), NBR2 full length (FL), T4, or T5 fragment expression vectors, and analyzed by Western blotting as indicated. (b) Protein lysates prepared from UMRC2 cells stably expressing EV or NBR2 #1 expression vectors were immunoprecipitated by IgG, LKB1 or Folliculin antibodies, and then were analyzed by Western blotting as indicated. Aliquots of the protein lysates (input) were also analyzed directly. (c) 786-O cells infected with either control shRNA or NBR2 shRNA were cultured in medium containing 0 or 25 mM glucose for 12 hours. Protein lysates were prepared and immunoprecipitated by IgG or LKB1 antibodies, and then were analyzed by Western blotting as indicated. Aliquots of the protein lysates (input) were also analyzed directly. (d) Empty vector (EV) or LKB1-infected Hela cells were transfected with EV or NBR2 #1 expression vectors. Protein lysates were prepared and analyzed by Western blotting as indicated. Unprocessed original scans of blots are shown in Supplemental Fig. 8.
Supplemental Figure 5 The working model of the reciprocal regulation between NBR2 and AMPK under energy stress, and its relevance to cancer development. See discussion for detailed description.
Supplemental Figure 6  NBR2 deficiency affects AMPK activation under long periods of energy stress. (a) MDA-MB-231 cells infected with either control shRNA or NBR2 shRNA were cultured in 0 mM glucose-containing medium for different hours, and protein lysates were prepared and analyzed by Western blotting. (b) MDA-MB-231 cells were cultured in 0 mM glucose-containing medium for different hours, and then subjected to real-time PCR analysis to measure NBR2 expression (Mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student’s t-test). Source data for b can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplemental Fig. 8.
Supplemental Figure 7 NBR2 deficiency does not affect BRCA1 expression. (a) MDA-MB-231 cells infected with either control shRNA or NBR2 shRNA were cultured in 25 or 0 mM glucose-containing medium for 24 hours, and then subjected to real-time PCR analysis to measure BRCA1 expression (Mean ± s.d., n=3 biologically independent extracts, two-tailed paired Student’s t-test). (b) Cell lysates were also analyzed by Western blotting as indicated. Source data for a can be found in Supplementary Table 1. Unprocessed original scans of blots are shown in Supplemental Fig. 8.
Supplemental Figure 8 Unprocessed scans of full blots.
Supplemental Figure 8 continued
Supplemental Figure 8 continued
Supplemental Figure 8 continued
Fig. 5

Supplemental Figure 8 continued
Supplemental Figure 8 continued
Supplemental Figure 8 continued
Fig. S4

Supplemental Figure 8 continued
Fig. S6

Fig. S7

Supplemental Figure 8 continued
Supplementary Table 1  Statistics source data.
Raw numbers (cell number) or normalized values of the indicated figure panels are provided.