The IncRNA Neat1 promotes activation of inflammasomes in macrophages

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The inflammasome has an essential function in innate immune, responding to a wide variety of stimuli. Here we show that the IncRNA Neat1 promotes the activation of several inflammasomes. Neat1 associates with the NLRP3, NLRC4, and AIM2 inflammasomes in mouse macrophages to enhance their assembly and subsequent pro-caspase-1 processing. Neat1 also stabilizes the mature caspase-1 to promote interleukin-1β production and pyroptosis. Upon stimulation with inflammasome-activating signals, Neat1, which normally resides in the paraspeckles, disassociates from these nuclear bodies and translocates to the cytoplasm to modulate inflammasome activation using above mechanism. Neat1 is also up-regulated under hypoxic conditions in a HIF-2α-dependent manner, mediating the effect of hypoxia on inflammasomes. Moreover, in the mouse models of peritonitis and pneumonia, Neat1 deficiency significantly reduces inflammatory responses. These results reveal a previously unrecognized role of IncRNAs in innate immunity, and suggest that Neat1 is a common mediator for inflammasome stimuli.
Inflammasomes are a group of multicomponent signaling platforms in the cytoplasm that control inflammatory response and anti-pathogen defense against a wide range of infection and damage signals. These signals, including pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), directly or indirectly activate one or more innate pattern recognition receptors (PRRs), which include nucleotide-binding domain (NBD) and leucine-rich repeat (LRR)-containing receptors (NRs, e.g., NLRP1, NLRP3, and NLRC4), cytosolic DNA sensors (AIM2), and Pyrin (also known as TRIM20). Upon activation, the sensor proteins bind to and induce the oligomerization of a common adaptor protein, apoptosis-associated speck-like protein containing CARD (ASC), leading to the formation of a single macromolecular aggregate known as ASC speck. Oligomerized ASC recruits pro-caspase-1, facilitating its auto-processing into the mature subunits. Active caspase-1 mediates proteolytic maturation of pro-inflammatory cytokines interleukin-1β (IL-1β) and IL-18 and elicits pyroptosis, a form of programmed cell death that exhibits features of both apoptosis (e.g., DNA fragmentation) and necrosis (e.g., plasma membrane rupture). While adequate inflammasome activation is crucial for the elimination of pathogens and damaged cells, dysregulation of inflammasome contributes to autoimmune, cancer, neurodegenerative disorders, and other diseases. Nevertheless, the regulation of inflammasomes is not well understood. Several inflammasomes respond to a limited set of signals. For example, the AIM2 and NLRC4 inflammasomes are assembled upon the sensing of double-stranded DNA (dsDNA) and specific bacterial proteins, respectively, while the AIM2 and NLRC4 inflammasomes are activated by an extraordinarily diverse array of PAMPs including several viral, bacterial, fungal pathogens, and DAMPs, such as crystalline, particulate (e.g., uric acid crystals, asbestos, and alum), extracellular ATP, pore-forming toxins, as well as change in cellular environment, notably hypoxia. A salient unresolved issue is how various inflammasomes collectively are able to respond to such a wide spectrum of stimuli.

The majority of transcripts transcribed from human or mouse genome are non-coding RNAs (lncRNAs), which are defined as transcripts longer than 200 nucleotides but lacking significant protein coding capacity. Thousands of lncRNAs have been identified to date, yet only a small fraction of them are characterized. In the context of innate immunity, a few lncRNAs have been implicated in regulation of inflammasome, including LinRNA-Gm4419, LincRNA-Cox2, and lincRNA-EPS. None have been reported to directly involve in inflammasome assembly.

In the current study, we examine lncRNAs that associate with NLRP3 inflammasome in mouse macrophages and identify Neat1 (nuclear enriched abundant transcript 1), a lncRNA transcribed from the multiple endocrine neoplasia locus (hence also known as Men). Neat1 and its human ortholog Neat1 maintain the structural integrity of the paraspeckles, a specific type of nuclear bodies in the interchromatin space whose function remains poorly understood. NEAT1 also regulates the expression of a group of chemokines and cytokines, including IL-6 and CXCL10, through the MAPK pathway. Of note, the expression of Neat1 is stimulated by many stimuli that also activate inflammasome, including infection of various viruses and some intracellular damages (e.g., ROS) that stabilize hypoxia-inducible factors (HIFs) and the tumor suppressor p53. Here we find that Neat1 promotes the activation of NLRP3, NLRC4, and AIM2 inflammasomes and enhances caspase-1 activation, cytokine production, and pyroptotic cell death. Mechanistically, Neat1 binds to pro-caspase-1 and facilitates the assembly of inflammasomes, and also stabilizes the mature caspase-1 and increases caspase-1 protease activity. In response to various inflammasome-activating signals, Neat1 is released from paraspeckles and translocated to the cytoplasm to participate in inflammasome activity. Our findings establish a direct role for lncRNAs in regulating inflammasomes and suggest that Neat1 may represent a downstream convergence point for inflammasome stimuli.

Results
Neat1 enhances the activation of the NLRP3 inflammasome. To investigate whether lncRNAs may regulate inflammasomes, we set out to identify lncRNAs that are associated with the NLRP3 inflammasome in murine immortalized bone marrow-derived macrophages (iBMDMs). We primed iBMDMs with lipopolysaccharides (LPS) and subsequently treated them with the potassium ionophore nigericin to activate the NLRP3 inflammasome. After treating these cells with UV to stabilize protein-RNA interactions, we immunoprecipitated NLRP3 inflammasomes using anti-NLRP3 antibody and analyzed the associated RNAs by high-throughput sequencing (GSE118722). Several non-coding RNAs were found to be enriched in NLRP3 inflammasomes, including those encoded by Ankrd52, Hipk3, Pou2f2, Neat1, Gm26917, Gm42418, Snrord34, Junos, Gm26767, and Gm23935 (Supplementary Fig. 1a). Among them were four lncRNAs: Gm26917, Gm42418, Gm26767, and Neat1 (Supplementary Fig. 1a).

To evaluate their effect on the NLRP3 inflammasome, we stably knocked down each of these four lncRNAs in iBMDMs using short hairpin RNA (shRNA). Knocking down Gm26917, Gm42418, or Gm26767 lncRNAs had no effect on the NLRP3 inflammasome (data not shown). Neat1 (Gene ID: 66961), is expressed in two isoforms, 3.2 kb Neat1_1 (NR_003513.3, also known as Men epsilon) and 20 kb Neat1_2 (NR_003513.3, also known as Men beta) (Supplementary Fig. 1b). We knocked down Neat1_1 and Neat1_2 simultaneously using two independent shRNAs (#1 and #2; Supplementary Fig. 1b, c). Interestingly, the knockdown of total Neat1 resulted in a strong reduction in oligomerization of ASC, indicative of impaired assembly of the inflammasome (Fig. 1a). This was accompanied by a noticeable reduction in the activation of caspase-1, processing of pro-interleukin 1β (pro-IL-1β) (Fig. 1a), and secretion of mature IL-1β (Fig. 1b, c). We also knocked down Neat1_2 along with an isoform-specific shRNA (Supplementary Fig. 1b, d), and observed an almost indistinguishable set of results (Fig. 1d, e). Conversely, we forced the expression of Neat1. Neat1_2 is too long to be readily cloned and expressed. Therefore, we used the short variant Neat1_1 (Supplementary Fig. 1b). When Neat1_1 was overexpressed in iBMDMs (Supplementary Fig. 1h), LPS/nigericin-induced ASC aggregation, pro-caspase-1 cleavage, and IL-1β secretion were all increased (Figs. 1f, g).

Knocking down total Neat1 or Neat1_2 alone, or overexpressing Neat1_1, did not alter the expression of NLRP3, ASC, pro-caspase-1, or pro-IL-1β (Figs. 1a, d, f and Supplementary Fig. 1b), suggesting that Neat1 promotes the assembly of the NLRP3 inflammasome rather than increasing the expression of its components or downstream targets. These changes in Neat1 levels did not influence the secretion of TNF-α either (Supplementary Fig. 1e–g, i), indicating a specific effect of Neat1 on NLRP3 inflammasome-mediated cytokine production.

To verify the stimulatory effect of Neat1 on the NLRP3 inflammasome, we generated Neat1 knockout (Neat1−/−) mice on the C57BL/6 background using CRISPR/Cas9-based genome engineering.
Fig. 1 Neat1 enhances the activation of the NLRP3 inflammasome. a–c Control iBMDMs and iBMDMs devoid of Neat1 by shRNA #1 (a, left and b) or #2 (a, right and c) were untreated, primed with LPS, or treated with nigericin after LPS priming. Whole cell lysates (WCL) and the supernatant (SN) and pellet (PE) fractions were analyzed by western blotting for indicated protein levels (a). Culture supernatant was analyzed by ELISA for IL-1β secretion (b, c).

d–g iBMDMs devoid of Neat1_2 (d, e) or overexpressing Neat1_1 (f, g) and the corresponding controls were treated with or without LPS or nigericin as indicated, and analyzed for protein levels (d, f) and IL-1β secretion (e, g). h iBMDMs from Neat1 wild-type (Neat1+/+) or knockout (Neat1−/−) mice were primed with LPS and then stimulated with nigericin as indicated. Protein levels (h) and IL-1β secretion (i) were analyzed. In b, c, e, g, and i, data shown are mean ± SD (n = 4). ***P < 0.001, two-tailed t-test. Source data are provided as a Source Data file.
edits SETK1.7, a pro-inflammatory cytokine that stimulates immune response. However, in IL-6^{−/−} and IL-6^{+/−} BMDMs (Supplementary Fig. 3a) treated with LPS and nigericin, both caspase-1 activation and IL-1β secretion occurred to a comparable extent (Fig. 3a and Supplementary Fig. 3b). Moreover, siRNA-mediated knockdown of total Neat1 (Supplementary Fig. 3c, d), or Neat1_2 alone (Supplementary Fig. 3e), similarly inhibited caspase-1 activation and IL-1β secretion in IL-6^{−/−} and IL-6^{+/−} BMDMs (Fig. 3b–d). Thus, IL-6 does not appear to mediate the function of Neat1 on the NLRP3 inflammasome.

Neat1 interacts with caspase-1 p20 domain. Given that Neat1 associates with, and promote the assembly of, the NLRP3 inflammasome in an IL-6-independent manner, we evaluated which component(s) of the NLRP3 inflammasome interacts with Neat1. NLRP3, ASC, and pro-caspase-1 were immunoprecipitated by their specific antibodies from lysates of murine iBMDMs at different stages of stimulation: no stimulation, primed with LPS only, and primed with LPS followed by stimulation with nigericin. Total Neat1 and Neat1_2 were found in pro-caspase-1 immunoprecipitates from cells of all three stages, but in NLRP3 and ASC immunoprecipitates only from cells that are activated by both LPS and nigericin (Fig. 4a–c and Supplementary Fig. 4a–c).

In a complementary approach, we pulled down Neat1 from cell lysates using biotinylated Neat1 antisense DNA probes, and analyzed the presence of NLRP3 inflammasome components in the pull-down samples. This again revealed that pro-caspase-1 bound to Neat1 in cells of all three stages, whereas ASC and NLRP3 bound to Neat1 only in cells treated with both LPS and nigericin (Fig. 4d and Supplementary Fig. 4d). Thus, Neat1 likely interacts with pro-caspase-1 constitutively and directly, and with NLRP3 and ASC inductively and indirectly when these components are incorporated into the inflammasome together with pro-caspase-1.

Likewise, during the activation of the NLRC4 and AIM2 inflammasomes, Neat1 bound to pro-caspase-1 directly in cells of all stages: non-stimulated, LPS-primed, and LPS-primed followed by flagellin or poly(dA:dT) stimulation, whereas Neat1 bound to NLRC4/AIM2 and ASC only upon flagellin-dependent NLRC4 inflammasome activation or poly(dA:dT)-dependent AIM2 inflammasome activation (Supplementary Fig. 4e–h).

Upon activation, caspase-1 is processed to the mature subunits p20 and p10. We observed that more p20 than p10 subunits were pulled down by the Neat1 antisense DNA probes (Fig. 4d). This observation suggested that Neat1 may directly associate with the p20 subunit of the mature caspase-1 or the region corresponding to p20 on pro-caspase-1, and indirectly with the p10 subunit within the assembled mature caspase-1 hetero-tetramer. To verify this, we generated a panel of caspase-1 deletion mutations, each tagged with the Flag epitope (Supplementary Fig. 5a, b). These mutants, as well as full-length pro-caspase-1, were expressing in 293T cells, and the cell lysates were incubated with biotin-labeled Neat1_1. This assay showed that Neat1_1 bound to pro-caspase-1 and the p20 region, but not the deletion of the CARD (ΔCARD) or the p10 region (Δp10), abolished the interaction with Neat1_1 (Fig. 4f). Therefore, the p20 region of pro-caspase-1 is both sufficient and necessary for the interaction with Neat1, and this interaction persists upon caspase-1 activation.

To map the caspase-1-binding region on Neat1, we performed NLRP3 CLIP-seq peaks analysis. A strong and specific peak in the 5′ region of Neat1_1 and Neat1_2, along with some other weak peaks in the rest of Neat1_1, was identified (Supplementary Fig. 5c), consistent with binding of total Neat1 and Neat1_2 with the NLRP3 inflammasome. We generated three Neat1_1 segments according to the CLIP-seq analysis (Supplementary Fig. 5c), and incubated each of them with HA-tagged pro-caspase-1. Only full-length Neat1_1 and segment 1, which contained the 5′ 1 kb region, could pull down HA-tagged pro-caspase-1 (Supplementary Fig. 5d), suggesting that Neat1 binds with pro-caspase-1 via its 5′ region.

Neat1 stabilizes the caspase-1 hetero-tetramers. The active caspase-1, an αββ hetero-tetramer formed by two p10 subunits and two p20 subunits, is turned over by the ubiquitin-proteasome pathway.42 The hetero-tetramer is also unstable and readily dissociates.43 Neat1_1 did not affect the ubiquitination of either pro-caspase-1 or the p20 subunit (Supplementary Fig. 6a). Instead, Neat1 increased the interaction between p20 and p10 (Fig. 5a, b and Supplementary Fig. 6b, c), suggesting that Neat1 stabilizes the (p20:p10)2 hetero-tetramer. Mature caspase-1 can also exist as a transient (p33:p10)2 species, where p33 contains...
**Fig. 2** Neat1 enhances the activation of NLRC4 and AIM2 inflammasomes. 

**a-d** iBMDMs devoid of Neat1 by shRNA #1 (a, b), iBMDMs overexpressing Neat1_1 (c, d), and the corresponding control iBMDMs were primed with LPS, and then stimulated with flagellin (a, c) or poly(dA:dT) (b, d). WCL and SN were analyzed by western blotting. 

**e-f** BMDMs from Neat1^{+/+} or Neat1^{−/−} mice were primed with LPS and then stimulated with flagellin (e) or poly(dA:dT) (f). WCL and SN were analyzed by western blotting. 

**g-h** Control iBMDMs and iBMDMs ectopically expressing Neat1_1 were primed with LPS and then stimulated with flagellin (g) or poly(dA:dT) (h). Cell lysates were immunoprecipitated with normal rabbit IgG or ASC antibody. The samples were analyzed by western blotting as indicated. Source data are provided as a Source Data file.
both the CARD and p20 regions\textsuperscript{44}. Of note, Neat1_1 could also increase the interaction between p33 and p10 (Fig. 5c, d and Supplementary Fig. 5d, e). Therefore, Neat1 stabilizes the subunit–subunit interactions of mature caspase-1 hetero-tetramers. Consistently, caspase-1 activity was significantly reduced in Neat1\textsuperscript{−/−} BMDMs compared to Neat1\textsuperscript{+/+} BMDMs upon the formation of NLRP3, NLRC4, or AIM2 inflammasomes (Fig. 5e), while caspase-1 activity was strongly increased in Neat1_1-overexpressing cells compared to control cells upon the formation of these canonical inflammasomes (Fig. 5f). Therefore, Neat1 strengthens the inter-subunit interactions within mature caspase-1 and augments the protease activity.

**Neat1 promotes caspase-1-dependent pyroptosis.** In addition to cytokine secretion, activation of caspase-1 by canonical inflammasomes results in pyroptosis\textsuperscript{45}. We observed significant cell death among LPS and nigericin co-stimulated iBMDMs, but not untreated or LPS-primed iBMDMs, as shown by propidium iodide-positive staining and lactate dehydrogenase (LDH) release. Overexpression of Neat1_1 noticeably augmented LPS/nigericin-induced pyroptosis (by \textasciitilde 120%; Fig. 6a, b), whereas Neat1 silencing dramatically reduced pyroptosis (by \textasciitilde 80%; Fig. 6c, d). Likewise, Neat1 knockout rendered BMDMs high resistant to pyroptosis (reduced by \textasciitilde 70%; Fig. 6e, f). Therefore, Neat1 promotes caspase-1-dependent pyroptotic cell death.

**Neat1 is exported to cytoplasm upon inflammasome activation.** Murine Neat1 and its human ortholog are highly abundant lncRNAs that are mainly localized to the nucleus of unstressed cells\textsuperscript{46}, while assembly of various canonical inflammasomes occurs in the cytoplasm\textsuperscript{47-48}. We found that, upon the stimulation of LPS and nigericin, the levels of total Neat1 in total cell lysates and supernatant fraction were analyzed by western blotting. b-d IL-6\textsuperscript{+/+} or IL-6\textsuperscript{−/−} BMDMs were transfected with control siRNA (b-d), Neat1 siRNA #1 (b), Neat1 siRNA #2 (c), or Neat1_2 siRNA (d). Twenty-four hours later, cells were stimulated with LPS and nigericin. WCL and SN of above cells were subjected to western blotting with indicated antibodies. Source data are provided as a Source Data file.

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**Fig. 3** The effect of Neat1 on the NLRP3 inflammasome activation is independent of IL-6. a BMDMs derived from IL-6 wild-type (IL-6\textsuperscript{+/+}) or knockout (IL-6\textsuperscript{−/−}) mice were primed with LPS and then treated with nigericin. Protein levels in total cell lysates and supernatant fraction were analyzed by western blotting. b-d IL-6\textsuperscript{+/+} or IL-6\textsuperscript{−/−} BMDMs were transfected with control siRNA (b-d), Neat1 siRNA #1 (b), Neat1 siRNA #2 (c), or Neat1_2 siRNA (d). Twenty-four hours later, cells were stimulated with LPS and nigericin. WCL and SN of above cells were subjected to western blotting with indicated antibodies. Source data are provided as a Source Data file.
Fig. 4 Neat1 interacts with caspase-1 p20 domain. 

**a**–**c** Cell lysates of normal iBMDMs, LPS-primed iBMDMs or LPS-nigericin-co-stimulated iBMDMs were incubated with normal rabbit IgG (**a**–**c**), NLRP3 antibody (**a**), ASC antibody (**b**), or caspase-1 antibody (**c**) for RIP. The immunoprecipitates were analyzed by real-time RT-PCR to examine enrichment efficiency of Neat1 and by Western blotting for protein levels. **d** Cell lysates of normal, LPS-primed, or LPS-nigericin-co-stimulated iBMDMs were incubated with biotin-labeled Neat1 sense or antisense DNA probes immobilized on beads. The precipitated samples were analyzed by real-time RT-PCR for Neat1 contents, and by Western blotting for indicated protein levels. In vitro transcribed biotin-labeled Neat1 was incubated with lysates extracted from 293T cells expressing Flag-tagged wild-type pro-caspase-1, CARD, p20, or p10 for 3 h. The input and biotin pull-down samples were then carried out to Western blotting.

In **a**, data shown are mean ± SD (n = 5). In **b** and **d**, data shown are mean ± SD (n = 3). In **c**, data shown are mean ± SD (n = 6). *P < 0.05, **P < 0.01, two-tailed t-test. Source data are provided as a Source Data file.
Fig. 5 Neat1 stabilizes the caspase-1 hetero-tetramers and increases caspase-1 activity. a-d 293T cells were infected with lentiviruses expressing control RNA or Neat1_1 and then co-transfected with Flag-p20 and HA-p10 (a, b) or Flag-p33 and HA-p10 (c, d). Cell lysates were incubated with anti-Flag mAb (M2) beads. Input samples and pull-down products were subjected to western blotting (a, c). Relative ratios of HA-p10 pulled down by Flag-p20 (b) or Flag-p33 (d) were shown in histogram, based on the data of three independent replication experiments. e Neat1<sup>+/+</sup> and Neat1<sup>−/−</sup> BMDMs were primed with LPS and then activated with nigericin, flagellin, or poly(dA:dT). Mature caspase-1 activity was measured. f Control or Neat1_1 overexpressing iBMDMs were primed with LPS and then activated with nigericin, flagellin, or poly(dA:dT). Mature caspase-1 activity were measured. In b, d, e and f, data shown are mean ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed t-test. Source data are provided as a Source Data file.
isoform remained unchanged (Supplementary Fig. 7a). To understand how Neat1 participates in the inflammasome activation, we examined the presence of Neat1 in the cytoplasmic fraction and culture medium of cells treated under different conditions. Compared with untreated or LPS-primed iBMDMs, LPS and nigericin co-stimulated iBMDMs contained more Neat1 in the cytoplasm (~8.5% versus ~1%; Fig. 7a and Supplementary Fig. 7b). Moreover, consistent with the increased pyroptosis, LPS and nigericin co-stimulated iBMDMs also released more Neat1 into the culture medium (Supplementary Fig. 7c). Similarly, more Neat1 was released from nucleus to cytoplasm in LPS/poly(dA:dT)-treated iBMDMs compared to untreated or LPS-primed iBMDMs (Fig. 7b, c and Supplementary Fig. 7d, e). Additionally, we treated iBMDMs with LPS and 2DG to stimulate the formation of Nlrp1b inflammasome. This also led to the translocation of Neat1 to the cytoplasm (Supplementary Fig. 7f, g). These observations suggest that Neat1 is released from nucleus to the cytoplasm during the activation of the NLRP3, NLRC4, AIM2, or Nlrp1b inflammasome.

Perturbation of paraspeckles leads to the release of Neat1. Neat1 is concentrated in paraspeckles and is an essential architectural component of these nuclear bodies35,36. To examine how LPS and nigericin co-stimulation may induce Neat1 nuclear export, we examined levels of three main paraspeckle proteins, NonO, Pspc1, and Sfpq35. Compared with control or LPS-treated iBMDMs, Pspc1 and the short isoform of Sfpq (Sfpq-S) declined in their abundance in LPS and nigericin co-treated iBMDMs, while the levels of NonO and the long isoform of Sfpq (Sfpq-L) remained unchanged (Fig. 8a). Levels of Pspc1 and Sfpq-S were also decreased in iBMDMs activated by flagellin or poly(dA:dT) (Fig. 8b, c). In addition, levels of Pspc1 declined in 2DG-activated iBMDMs (Supplementary Fig. 8a). Accordingly, the size and number of paraspeckles were dramatically reduced in LPS and nigericin co-stimulated iBMDMs compared to untreated or

Fig. 6 Neat1 promotes caspase-1-dependent pyroptosis. a–f Neat1-overexpressing iBMDMs (a, b), Neat1-knockdown iBMDMs (c, d), Neat1−/− BMDMs (e, f), and the corresponding control cells were primed with LPS and then stimulated with nigericin. Cells were stained with PI and analyzed under microscope (a, c, and e). Percentages of pyroptotic cells were measured by LDH release into supernatants (b, d, and f). In a, c, and e, the bar graph indicates 300 μm. In b, d, and f, data shown are mean ± SD (n = 4). ***P < 0.001, two-tailed t-test. Source data are provided as a Source Data file.
lPS-primed iBMDMs (Fig. 8d and Supplementary Fig. 8b, c). Consistently, the interaction of Nono with Pspc1 and Spq was reduced in response to the activation of the NLRC4, AIM2, and Nlrp1b inflammasome (Fig. 8e, f and Supplementary Fig. 8d). All above results claim that activation of various inflammasomes weakens assembly of paraspeckles, which store Neat1 in nucleus.

To verify that the perturbation of paraspeckles results in the translation of Neat1 to the cytoplasm, we knocked down Nono, Pspc1, and Spq individually (Supplementary Fig. 8e, f). In each case, the nuclear export of both total Neat1 and Neat_2 was observed, and a substantial fraction of Neat1 (~15–20%) was found in the cytoplasm (Fig. 8g, h). Therefore, stimuli, that activate various inflammasomes, disrupt paraspeckles leading to the translocation of Neat1 to the cytoplasm, where Neat1 influences the assembly of inflammasomes.

Neat1 mediates the effect of HIF-2α on inflammasomes. The expression of Neat1 is induced by infection of viruses (e.g., influenza virus and herpes simplex virus)49,50, and is also regulated by the tumor suppressor p5351, a central sentinel for various internal and external stresses32,53. In addition, hypoxia, which promotes inflammation via HIF-1α-mediated induction of NLRP3 and IL-1β24,25, can induce Neat1 through HIF-2α-mediated transcriptional activation34,35. Thus, Neat1 may function as a stress sensor for associated inflammasomes. To test this possibility, we investigated the role of Neat1 in hypoxia-mediated activation of the NLRP3 inflammasome. iBMDMs cultured under hypoxic conditions exhibited increased levels of Neat1 (Supplementary Fig. 9a), HIF-1α, HIF-2α, p65, NLRP3, and IL-1β (Fig. 9a), which was accompanied by enhanced NLRP3 inflammasome formation, caspase-1 activation, and IL-1β secretion (Fig. 9a). However, knockout of Neat1 not only reduced the activation of NLRP3 inflammasome under normoxic conditions, but also effectively prevented the increase of inflammasome activation under hypoxic conditions (Supplementary Fig. 9b), underscoring a critical role for Neat1 in hypoxia-induced NLRP3 inflammasome activation. In addition, activation of NLRC4 and AIM2 inflammasomes was also increased under hypoxic conditions, along with increased expression of HIF-1α, HIF-2α, and IL-1β (Fig. 9a, b). Knocking down either HIF-1α (Fig. 9d, f, h) or HIF-2α (Fig. 9e, g, i) reduced hypoxia-induced caspase-1 activation and IL-1β maturation under the condition of NLRP3, NLRC4, or AIM2 inflammasome activation (Fig. 9d–i), indicating that both HIF-1α and HIF-2α mediate the effect of hypoxia on canonical inflammasomes. HIF-1α knockdown, but not HIF-2α knockdown, reduced the activation of diverse inflammasomes in iBMDMs devoid of Neat1 (Fig. 9d–i), which claims that Neat1-silencing could efficiently block the effect of HIF-2α, but not HIF-1α, on inflammasomes activation. These results suggest that hypoxia-induced activation of the NLRP3, NLRC4, and AIM2 inflammasomes and associated inflammatory responses are in part due to HIF-2α-mediated upregulation of Neat1.

Loss of Neat1 reduces inflammation in vivo. To investigate the effect of Neat1 in vivo, we used an NLRP3 inflammasome-mediated, IL-1-dependent mouse peritonitis model induced by the aluminum salt alum36. We intraperitoneal (i.p.) injected alum in Neat1+/− and Neat1−/− mice to induce peritonitis, and assayed IL-1β in the lavage fluid. A significant reduction of IL-1β secretion in the lavage fluid was observed in Neat1−/− mice compared to Neat1+/− mice (by ~67%; Fig. 10a). Moreover, collected peritoneal exudate cells (PECs) and analyzed alum-induced recruitment of

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**Fig. 7** Neat1 is exported from nucleus to cytoplasm in response to inflammasome-activating stimuli. a–c Untreated iBMDMs (a–c), LPS-primed iBMDMs (a–c), nigericin-activated iBMDMs (a), flagellin-activated iBMDMs (b), or poly(dA:dT)-activated iBMDMs (c) were subjected to fractionation into cytoplasmic and nuclear extracts. Cytoplasmic RNA was analyzed for the Neat1 expression by real-time RT-PCR. d The subcellular co-localization of Neat1 (green) and ASC (purple) in untreated, LPS-primed or LPS-nigericin-co-stimulated iBMDMs was analyzed by RNA-FISH and IF. In d, the bar graph indicates 5 μm. In a, b, and c, data shown are mean ± SD (n = 3). *P < 0.05, **P < 0.001, two-tailed t-test. Source data are provided as a Source Data file.
inflammatory cells by flow cytometry (Supplementary Fig. 10a). The number of total PECs recruited upon alum challenge was markedly decreased by 58% in Neat1−/− mice (Fig. 10b). Alum-induced recruitment of neutrophils and Ly6C+ monocytes was also strongly inhibited in the Neat1−/− mice (Fig. 10c, d).

To evaluate the role of Neat1 on NLRC4 inflammasome activation in vivo, we used a mouse model of flagellin-induced pneumonia. Neat1+/+ and Neat1−/− mice were intranasally instilled with TurboFect reagent and flagellin to induce pneumonia. We found that IL-1β secretion in bronchoalveolar...
Fig. 9 Neat1 mediates the effect of HIF-2α on inflammasomes under hypoxic condition. a–c iBMDMs cultured under normoxic or hypoxic conditions were untreated (a–c), primed with LPS (a–c), or co-stimulated with LPS and nigericin (a), flagellin (b), or poly(dA:dT) (c). WCL (a–c), SN (a–c), and PE (a) were subjected to western blotting. d–i Control (d–i), HIF-1α-silenced (d, f, and h), HIF-2α-silenced (e, g, and i), Neat1-silenced (d–i), HIF-1α-Neat1-double-silenced (d, f, and h), or HIF-2α-Neat1-double-silenced (e, g, and i) iBMDMs were cultured under hypoxic conditions and primed with LPS, followed by stimulation with nigericin (d, e, f, g, and i), flagellin (f, g, or poly(dA:dT) (h, i). WCL and SN were analyzed by western blotting. Source data are provided as a Source Data file.
**Fig. 10** Loss of Neat1 impairs alum-induced peritoneal inflammation and attenuates flagellin-induced lung inflammation in vivo. 

**a** IL-1β content in the lavage fluid from Neat1+/+ or Neat1−/− mice 12 h after PBS or alum injection. 

**b** Absolute numbers of Neat1+/+ or Neat1−/− PECs recovered 12 h after PBS or alum injection. 

**c, d** Absolute numbers of neutrophils (c) and Ly6C+ monocytes (d) exuded to the peritoneum 12 h after PBS or alum injection. 

**e** IL-1β content in the BALF from Neat1+/+ or Neat1−/− mice 12 h after isotonic saline or flagellin intranasal instillation. 

**f** Absolute numbers of Neat1+/+ or Neat1−/− PECs recovered 12 h after isotonic saline or flagellin intranasal instillation. 

**g, h** Absolute numbers of neutrophils (g) and Ly6C+ monocytes (h) exuded to the lung 12 h after isotonic saline or flagellin intranasal instillation. In **a**–**h**, data shown are mean ± SD (n = 6). *P < 0.05, **P < 0.01, ***P < 0.001, two-tailed t-test. 

Source data are provided as a Source Data file.
lavage fluid (BALF) was decreased by 70% in flagellin-challenged Neat1−/− mice compared to flagellin-challenged Neat1+/+ mice (Fig. 10e). We also analyzed bronchoalveolar lavage cells (BALCs) and flagellin-induced recruitment of inflammatory cells by flow cytometry (Supplementary Fig. 10b). The total number of BALCs recruited upon flagellin challenge was decreased by 70% in Neat1−/− mice (Fig. 10f). Furthermore, flagellin-induced recruitment of neutrophils and Ly6C+ monocytes was strongly decreased in Neat1−/− mice (Fig. 10g, h).

The overt defects of Neat1−/− mice in alumin-induced peritoneal inflammation and flagellin-induced lung inflammation emphasize an important function for Neat1 in the activations of the canonical inflammasomes, the subsequent accumulation of immune cells and the associated symptoms in vivo.

Discussion

Proper control of inflammasome assembly and caspase-1 activation permits effective antimicrobial and inflammatory responses while avoiding tissue damage. Yet, how these supramolecular assemblies are regulated remains incompletely defined. LncRNAs are increasingly recognized as important regulators in different biological settings, but few lncRNAs are implicated in innate immune cells and the associated symptoms in vivo.55,61

The current work reveals that the lncRNA Neat1 plays a critical role in regulating various caspase-1-associated canonical inflammasomes including NLRC4, AIM2, and especially NLRP3 inflammasomes. Neat1 promotes the assembly of inflammasomes, and also stabilizes the mature caspase-1 tetramers, (p20:p10), and (p33:p10), and increases their protease activity. Thus, Neat1 is likely a previously unanticipated RNA component that enables the assembly of these largely proteinaceous signaling platforms to facilitate the recruitment, maturation, and stabilization of caspase-1 in the activated macrophages. The ability of Neat1 to directly regulate inflammasomes provides a notable example for the role of lncRNAs in regulating large multimeric protein complexes in the cytoplasm.

The assembly of inflammasomes requires a common priming event, such as LPS-induced activation of TLRs, which induces the expression of NLRP3 and pro-IL-1β. However, the NLRC4, AIM2, and especially NLRP3 inflammasomes are versatile inflammasome, collectively responding to a wide range of stimuli.52,46,61-64 It remains unclear whether these stimuli also employ a general mechanism to modulate inflammasomes. Of note, the expression of Neat1 is highly regulated by various signals, including those that activates the tumor suppressor p53 (refs. 40,51). Moreover, hypoxia is a key physiological stressor present within both normal and tumor microenvironments and a critical determinant of the immune response. Here we show that Neat1 links the effect of hypoxia with the activation of NLRP3, NLRC4, and AIM2 inflammasomes. Both human Neat1 and murine Neat1 are thought to maintain the structural integrity of the paraspeckles, the function of which remains unclear. We find that the paraspeckles are disrupted in response to various inflammasome-activating signals, leading to the release of Neat1 and subsequent translocation to the cytoplasm, implying that paraspeckles may serve as a storage depot for Neat1. Therefore, the upregulation and translocation of Neat1 may be a converging event leading to the activation of the various caspase-1-associated canonical inflammasomes by a wide range of stimuli, under-scoring a common mechanism for the assembly of these inflammasome signaling platforms. In addition, our finding, along with previous observation that ASC and NLRP3 are recruited from other cellular components to mitochondria and associated endoplasmic reticulum membranes upon both priming and activation processes, also highlights the trafficking of inflammasome components as a major mechanism for the regulation of inflammasome activity.

Finally, by generating Neat1 knockout mice and analyzing their response to alumin-induced peritoneal inflammation and flagellin-induced lung inflammation, we demonstrate that Neat1 enhances the activation of NLRP3 and NLRC4 inflammasomes and promotes pyroptosis in vivo (Supplementary Fig. 11). Therefore, Neat1 may represent a therapeutic target for inflammasome-associated diseases such as gout and autoinflammatory syndromes.

Methods

Antibodies and reagents. Antibodies used for western blotting included anti-IL-1β (R&D Systems, 1:1000, AF-401-NA), anti-HIF-2α (R&D Systems, 1:1000, AF2997), anti-NLRP3 (Adipogen, 1:1000, AG-20B-0014), anti-ASC (Adipogen, 1:1000, AG-25B-0006), anti-caspase-1 (p20) (Adipogen, 1:1000, AG-20B-0042), anti-caspase-1 (p10) (Adipogen, 1:1000, AG-20B-0044), anti-p65 (Cell Signaling Technology, 1:1000, 8242), anti-HIF-1α (Cell Signaling Technology, 1:1000, 14179), anti-AIM2 (Cell Signaling Technology, 1:1000, 13095), anti-IL-6 (Cell Signaling Technology, 1:1000, 12912), anti-Actin (CMC-TAG, 1:2000, AT0009), anti-FLAG (Sigma-Aldrich, 1:2000, F3165), anti-Pspc1 (Santa Cruz, 1:200, sc-371481), anti-Sfpq (Santa Cruz, 1:200, sc-277196), anti-Nono (Abclonal, 1:500, A5282), anti-NLR4 (Abclonal, 1:500, A7382), anti-histone H2A (abcam, 1:1000, ab177308), HRP-linked rabbit IgG (Cell Signaling Technology, 1:1000, 7074), HRP-linked anti-mouse IgG (Cell Signaling Technology, 1:1000, 7076), HRP-linked anti-goat IgG (Santa Cruz, 1:1000, sc-2354). Antibodies used for immunoprecipitation included anti-NLRP3 (Cell Signaling Technology, 1:200, 15101), anti-caspase-1 (p20) (Adipogen, 1:200, AG-20B-0042), anti-ASC (Adipogen, 1:200, AG-25B-0006), anti-Nono (Abclonal, 1:50, A5282), Alexa fluor 488 donkey anti-rabbit IgG (H+L) (Life Technologies, 1:1000, A21206), Alexa fluor 568 donkey anti-mouse IgG (H+L) (Life Technologies, 1:1000, A10397), and Alexa fluor 647 donkey anti-rabbit IgG(H + L) (Life Technologies, 1:1000, A31573) were used for immunofluorescence. PerCP-Cy5.5 anti-mouse CD11b (BD Biosciences, 1:1000, 550993), APC anti-mouse Ly-6G (BD Biosciences, 1:1000, 560599), and PE anti-mouse Ly-6C (BD Biosciences, 1:1000, 560592) were used for flow cytometry. LPS, nigericin, flagellin, and poly(dA:dT) were purchased from Invivogen; Lipofectamine 2000 Transfection Reagent was from Invitrogen; DOTAP Liposomal Transfection Reagent was from Roche; puromycin, MG132, propidium iodide, and 2-deoxy-D-Glucose were from Solarbio; proteinase K and RiboLock were from Thermo Fisher Scientific; and Igepal CA-630 was used from Sigma. Caspase-1 Activity Assay Kit purchased from Beyotime was used to perform the semi-quantitative analysis of mature caspase-1 activity.

Mice. Neat1−/− mice on a C57BL/6 background were generated by Biocytogen Biological Technology Co., Ltd. Briefly, C57BL/6 mice and Kunming (KM) mice were housed in a specific pathogen free facility. For Neat1 targeting, two sgRNAs (Supplementary Table 1) were designed to target either a region upstream or downstream of the gene Neat1. For each targeting site, candidate guide RNAs were designed by the CRISPR design tool (http://crispr.mit.edu). Guide RNAs were screened on on-target activity using UCA1M. UCA1M (Universal CRISPR Activity Assay), a sgRNA activity detection system developed by Biocytogen, is simpler and more sensitive than MSDase assay. The Cas9 mRNA and sgRNAs were transcribed by T7 RNA polymerase in vitro. For Cas9 mRNA and sgRNAs production, the T7 promoter sequence to the Cas9 or sgRNA template by PCR amplification. The T7-Cas/sgRNA PCR products was gel purified and used as the template for in vitro transcription using the MEGA shortscript T7 kit (Life Technologies) according to the normal protocol. Purify the Cas9 mRNA and sgRNAs using the MEGAadent kit and elute with RNase-free water. C57BL/6 female mice and KM mouse strains were used as embryo donors and pseudopregnant foster mothers, respectively. Superovulated female C57BL/6 mice (3–4 weeks old) were mated to C57BL/6 stud males, and embryos were collected from the ampullae of super-ovulated female. Different concentrations of Cas9 mRNA and sgRNAs were mixed and co-injected into the cytoplasm of one-cell stage fertilized eggs. After injection, surviving zygotes were transferred into eutodirs of KM albino pseudopregnant females. Sixty-five pups were born and then identified by genotyping with tail DNA. Neat1−/− mice were used furtherly to reproduce the next generation, which includes Neat1−/−/Neat1−/− mice. IL-1β−/− mice were provided by professor Zexong Lian’s laboratory. Genotyping PCR primers sequences are shown in Supplementary Table 1. All animal experiments were undertaken and conducted with approval from the Animal Research Ethics Committee of the University of Science and Technology of China (Permit Number: USTC/ACUC1801057).
Cells at different stages were analyzed by immunoblotting.

Cell stimulation. Macrophages were cultured in DMEM complemented with 10% FBS, 1 mM sodium pyruvate, and 2 mM L-glutamine overnight, and the medium was changed to opti-MEM in the following day. Cells were primed with LPS (100 ng/mL) for 3 h, and subsequently stimulated with nigericin (5 μM) for 30 min, permeabilized with 0.5% Triton X-100 in PBS for 15 min, rinsed, blocked with 5% BSA in PBS for 30 min, and then incubated with indicated antibodies overnight at 4 °C. The next morning, cells were washed three times with PBS, and were recognized with indicated fluorescence-labeled secondary antibodies. After extensive washing with PBS, cells were imaging.

Enzyme-linked immunosorbent assay. Supernatants from cell culture were assayed for mouse IL-1β and mouse TNF-α according to manufacturer’s instructions of ELISA kits (R&D).

Cytosolic and nuclear fractionation. Indicated cells were incubated with hypotonic buffer (25 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 5 mM KCl) on ice for 5 min. An equal volume of hypotonic buffer containing 1% NP-40 was then added, and each sample was left on ice for another 5 min. After centrifugation at 5000 × g for 5 min, the supernatant was collected as the cytosolic fraction. The pellets were resuspended in nuclear resuspension buffer (20 mM HEPES, pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF) and incubated at 4 °C for 30 min. Nuclear fraction was collected after removing insoluble membrane debris by centrifugation at 12,000 × g for 10 min.

Propidium iodide staining and imaging. To examine pyroptotic cells, cells were treated as indicated in 12-well plates for image capture. Propidium iodide (1 μg/mL) was added to the medium for monitoring cell membrane integrity. Bright-field images of pyroptotic cells were captured using an Olympus IX71.

Immunoprecipitation and RNA pull-down. For immunoprecipitation assay, the lysates of iBMDMs were incubated with 2 μg/ml antibody recognizing particular protein of interest. Cells were washed with PBS, and then with 20 μl protein A/G magnetic beads for 1 h. After washing three times with lysis buffer (50 mM Tris–HCl [pH 7.4], 100 mM NaCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1/250 volume of Roche Protease Inhibitor Cocktail, 40 μM RNase inhibitor, and 20 μM MgCl₂) for 30 min, cell lysates were preclarified with protein A/G beads, and then were incubated with protein A/G beads coated with 15 μM NLRP3 antibody at 4 °C for 3 h. After extensive washing with washing buffer (50 mM Tris–HCl [pH 7.4], 100 mM NaCl, 0.2% Igepal CA-630, 0.1% SDS, 0.5% sodium deoxycholate, 40 μM RNase inhibitor), the bead-bound immunocomplexes were eluted using elution buffer (50 mM Tris–HCl, pH 8.0, 1% SDS, and 10 mM EDTA) at 65 °C for 10 min. To isolate protein-associated RNAs from the eluted immunocomplexes, samples were treated with proteinase K, and RNAs were extracted by phenol/chloroform. The RNA sequencing was performed and analyzed by KangChen Bio-tech, Shanghai, China. The sequencing data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE118722). Visualization analysis was performed by Integrative Genomics Viewer (IGV).

Flow cytometry. PECS and BALCs were obtained and blocked in PBS with 20% rat serum and 2 μg/ml mouse Ly-6C antibody for 1 h. After washing three times with PBS, cells were incubated with PerCP-CyTOM5.5 mouse CD11b antibody, APC-mouse Ly-6G antibody, and PE-mouse Ly-6C antibody for 1 h. After washing three times with PBS, stained cells were analyzed on the BD FACSCalibur to detect the proportion of neutrophils or Ly-6C⁺ monocyes. The flow cytometry data were analyzed on FlowJo software with the gating strategies indicated in Supplementary Fig. 10.

shRNAs and siRNAs. Forward (F) and reverse (R) DNA fragments (Supplementary Table 1) were annealed and then cloned into plasmid pLKO.1, which was used for gene knockdown in iBMDMs. siRNAs were used for gene knockdown in BMDMs. siRNAs’ target sequences are shown in Supplementary Table 1.

All relevant data are available from the corresponding authors upon reasonable request. The source data underlying all reported averages in Figs. 1b, c, e, g, h, 2a–d, 3a, 3b, 4a, 4b, 5a, 5b, 6a, 6b, 6c, 7a, 7b, 8a, 9a, 9b, 9c, and 9d are shown in Supplementary Data File. The source data underlying all uncropped versions of the blots and gels presented in Figs. 1a, 2a, b, 2c, 3a, 3d, 4a, 4f, 5a, 5c, 6a, and 6c.
Supplementary Figs. 1j, m, 2a, b, 3a, 4f, 5d, 6a–c, 8a, d, e, 9b are also provided in Source Data File. The sequencing data were deposited in the National Center for Biotechnology Information Gene Expression Omnibus database (GSE18722).

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Author contributions

P.Z., L.C., X.Y. and M.W. designed research; P.Z. and L.C. performed experiments and analyzed data; R.Z. helped to initiate this project and provided material support; P.Z., X.Y. and M.W. wrote and revised the manuscript.

Additional information

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