Loss of the abundant nuclear non-coding RNA MALAT1 is compatible with life and development

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Abbreviations: CLSM, confocal laser scanning microscopy; HCC, hepatocellular carcinoma; HP1, heterochromatin protein 1; KO, knockout; IncRNA, long non-coding RNA; MALAT1, metastasis-associated lung adenocarcinoma transcript 1; NEAT1, nuclear enriched abundant transcript 1; NSCLC, non-small cell lung cancer; Pc2: Polycomb 2 protein; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; RNAP, RNA polymerase; RPLP0, ribosomal protein, large subunit, P0; TRF2, telomere repeat factor 2; UBF, upstream binding factor; WT, wild-type; ZFN, zinc finger nuclease

The metastasis-associated lung adenocarcinoma transcript 1, MALAT1, is a long non-coding RNA (lncRNA) that has been discovered as a marker for lung cancer metastasis. It is highly abundant, its expression is strongly regulated in many tumor entities including lung adenocarcinoma and hepatocellular carcinoma as well as physiological processes, and it is associated with many RNA binding proteins and highly conserved throughout evolution. The nuclear transcript MALAT1 has been functionally associated with gene regulation and alternative splicing and its regulation has been shown to impact proliferation, apoptosis, migration and invasion.

Here, we have developed a human and a mouse knockout system to study the loss-of-function phenotypes of this important ncRNA. In human tumor cells, MALAT1 expression was abrogated using Zinc Finger Nucleases. Unexpectedly, the quantitative loss of MALAT1 did neither affect proliferation nor cell cycle progression nor nuclear architecture in human lung or liver cancer cells. Moreover, genetic loss of Malat1 in a knockout mouse model did not give rise to any obvious phenotype or histological abnormalities in Malat1-null compared with wild-type animals. Thus, loss of the abundant nuclear long ncRNA MALAT1 is compatible with cell viability and normal development.

Introduction

Recent deep transcriptome sequencing and tiling array studies have uncovered that between 70% and 90% of the human genome are estimated to be pervasively transcribed into mostly non-protein-coding RNA while only less than 2% of the human genome are estimated to be pervasively transcribed into mostly non-coding RNA while only less than 2% of the human genome is compatible with life and development. Some of these non-coding RNAs (ncRNAs) comprise small RNAs such as microRNAs as well as long non-coding RNAs (lncRNAs). However, only a minute fraction of the large number of non-coding gene products has been identified or characterized at all. The few individual examples studied in greater detail provide evidence that IncRNAs can execute a broad range of important functions in the cell. Individual long ncRNAs have been implicated e.g., in gene regulation, splicing control or X chromosome dosage compensation. Notably, some of the IncRNAs have also been implicated in human diseases and most importantly in cancer where IncRNAs can be deregulated or actively contributing to tumorigenesis.

One of the first IncRNA genes discovered was MALAT1, the metastasis-associated lung adenocarcinoma transcript 1, later also referred to as NEAT2 for nuclear-enriched abundant transcript 2. MALAT1 is highly abundant and is expressed in many healthy organs, most strongly in pancreas and lung. Deregulation or a functional role for MALAT1 have now been established in many human cancer entities including lung cancer, hepatocellular carcinoma (HCC), uterine endometrial stromal sarcoma, cervical cancer, breast cancer, osteosarcoma and colorectal cancer, but MALAT1 has also been linked to viral infection or alcohol abuse. In non-small cell lung cancer (NSCLC), MALAT1 is significantly associated with metastasis and serves as an independent prognostic marker for patient survival in early stage lung adenocarcinoma or squamous cell carcinoma of the lung. It promotes cell motility of lung cancer cells and supports...
proliferation and invasion of cervical cancer cells by regulating CASPASE-8, CASPASE-3, BAX, BCL-2 and BCL-XL. Furthermore, MALAT1 is functionally important for trophoblast invasion during embryonic development and is associated with synaptogenesis. In summary, MALAT1 is strongly expressed in many tissues, significantly regulated under various physiological and pathological conditions and has been linked to a plethora of functions at the cellular level including proliferation, apoptosis, migration or invasion.

At the molecular level, multiple functions have been proposed for MALAT1. MALAT1 is retained in the nucleus and specifically localizes to nuclear SC35 speckles that play a role in pre-mRNA processing. MALAT1 might regulate alternative pre-mRNA splicing by modulating the phosphorylation levels of serine/arginine splicing factors. Depletion of MALAT1 might alter the pre-mRNA processing of tissue factor and endoglin transcripts. Importantly, MALAT1 might interact with the polycomb repressive complex 1 (PRC1) and thus controls the relocation of growth control genes between polycomb bodies and interchromatin granules, places of silent or active gene expression, respectively. Additionally, MALAT1 RNA is frequently found in many PAR-CLIP experiments as a common interaction partner of RNA binding proteins potentially indicating further functions of MALAT1. Also, the MALAT1 transcript can be processed into a tRNA-like small cytoplasmic RNA, the mascRNA, which might fulfill additional, so far unknown functions. Given all of these divergent potential activities, MALAT1 could function in a cell type- or tissue-specific manner despite its ubiquitous expression. Finally, MALAT1 is highly conserved throughout evolution across many mammalian species, underscoring its potential functional importance.

In summary, these various lines of evidence culminate in the hypothesis that MALAT1 must be an important and most likely essential gene given its high and ubiquitous abundance, its specific regulation and localization patterns, its evolutionary conservation, its many interaction partners, molecular mechanisms and the strong impact of its deregulation on many cellular phenotypes including fundamental processes such as proliferation, migration or invasion.

The functional characterization of lncRNAs has greatly been hampered by a lack of quantitative loss-of-function models. Many lncRNAs—especially if they are nuclear, highly abundant or highly structured—can only be ineffectively targeted by RNA interference. In particular, this is a concern for studies of the highly abundant MALAT1, which was reduced only two- to 4-fold by shRNAs or siRNAs. Accordingly, these approaches make it difficult to distinguish between specific and off-target effects and their results may be prone to false-negative results due to an insufficient knockdown. Genetic knockout models have only been established for very few lncRNAs. For example, the Malat1-neighboring IncRNA Neat1 (nuclear enriched abundant transcript 1), which is essential for paraspeckle formation, has been knocked out in mice, but no phenotype has been reported so far. To overcome this challenge and to create quantitative loss-of-function models, we have developed a strategy to generate functional IncRNA knockouts also in human cell lines using zinc finger nucleases to stably integrate RNA destabilizing elements into the human genome site-specifically at the start of an IncRNA gene.

Here, we exploit this technique and achieve the efficient silencing of MALAT1 expression in human lung and liver cancer cells. Surprisingly, we demonstrate that the lncRNA MALAT1 is not essential for cell proliferation, cell cycle progression or maintenance of nuclear architecture. Our human loss-of-function model is complemented by a Malat1 knockout mouse model. Malat1-null mice are viable and without any histological signs of developmental defects. Thus, the loss of the highly abundant, nuclear enriched and evolutionarily conserved lncRNA MALAT1 is unexpectedly compatible with life and development.

Results

Depletion of MALAT1, a highly conserved and abundant lncRNA, from human cancer cells. As a first indication for the putative functional relevance of MALAT1, we verified its evolutionary conservation, strong expression and regulation. The lncRNA MALAT1 displayed a high level of sequence conservation throughout 33 mammalian species (Fig. 1A). In human cells, MALAT1 showed a ubiquitous expression, as it is detected in immortalized primary human cells (1°F and HEK293) as well as in a broad range of cancer cell lines derived from different tissues (Fig. 1B, left panel). In addition, MALAT1 was highly abundant and even exceeded the expression of classical housekeeping genes like for example RPLP0 (ribosomal protein, large, P0) (Fig. 1B right panel).

Based on this pattern of conservation and high expression, we aimed for unraveling the role of MALAT1 in cancer cells as well as in normal development. Therefore, we employed an innovative approach that we recently developed to create highly specific and efficient loss-of-function models of abundant ncRNAs in human cancer cell lines. This approach relies on the stable and site-specific integration of RNA destabilizing elements into the cancer cell genome mediated by zinc finger nucleases (ZFNs) (Fig. 1C). The ZFN binds to a specific site in the genome and introduces a DNA doublestrand break. When a repair template is present, this break is repaired via homologous recombination allowing a site-specific integration of exogenous sequences. Here, we integrate a cassette comprising the green fluorescent protein (GFP) as a selection marker and a polyadenylation signal (polyA), which functions as the RNA destabilizing element silencing downstream sequences.

Previously, we had only generated A549 bulks of lung adenocarcinoma cells deficient of MALAT1. Here, we extended the loss-of-function model to the liver cancer line HLE (hepatocellular carcinoma). As in A549 cells, MALAT1 was specifically and very efficiently suppressed in stable HLE lines. We obtained single cell MALAT1 knockout clones (KO) in both cancer cell lines with a 1,000-fold reduction of MALAT1 expression in A549 KO clones and over 200-fold reduction of MALAT1 in HLE KO clones (Fig. 1D). This approach depleted the full-length MALAT1 transcript: two different qRT-PCR primer pairs
Figure 1. For figure legend see page 1079.
Profiles did not significantly differ between WT and KO clones with similar fractions of cells in G1-, S- and G2/M-phase, respectively (Fig. 2B and D). Thus, complete loss of MALAT1 did not impact cell proliferation or cell cycle progression in the lung and liver cancer cells studied here.

Loss of MALAT1 does not affect the global nuclear architecture. A number of studies point to a crucial role of RNA as an architectural factor for shaping the genome and its nuclear environment. Since MALAT1 is a highly abundant RNA strongly enriched in the nucleus, we hypothesized that its loss might affect the structural organization of the nucleus. Accordingly, we examined the effect of the MALAT1 knockout with respect to several nuclear subcompartments for which an architectural role of RNA has been reported previously. RNA-dependent changes in nuclear morphology at a resolution of 200–300 nm can be directly detected after fluorescent labeling via evaluation of optical sections acquired by confocal laser scanning microscopy located at either end of the MALAT1 transcript yielded the same negative results for MALAT1 expression in the knockout cell clones (Fig. 1D).

MALAT1 is not critical for lung or liver cancer cell proliferation. One of the most prominent characteristics of cancer cells is their ability to proliferate—even in the absence of external stimuli due to deregulated signaling cascades. We used our knockout system to analyze the role of MALAT1 in lung and liver cancer cell proliferation. Therefore, we performed cell cycle analyses and proliferation assays with a panel of three MALAT1 wild-type (WT) control cell lines and three KO clones per cell line (Fig. 2A–D). As control cells, we included the parental cell lines (A549; HLE) and two WT single cell clones for each cell line that had undergone clonal selection at the same time as the KO clones. We compared the proliferation between WT and KO clones, but could not detect any significant differences in bromodeoxyuridine assays (Fig. 2A and C). Additionally, the cell cycle profiles did not significantly differ between WT and KO clones with similar fractions of cells in G1-, S- and G2/M-phase, respectively (Fig. 2B and D). Thus, complete loss of MALAT1 did not impact cell proliferation or cell cycle progression in the lung and liver cancer cells studied here.

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As discussed previously, a number of proteins that fulfill architectural functions for the formation of heterochromatic regions at the pericentromeres, centromeres and telomeres are targeted to these chromosomal loci via RNA. Accordingly, we investigated...
Thus, loss of \textit{MALAT1} had no detectable impact on the nuclear structures analyzed here.

\textbf{Generation and analysis of \textit{Malat1}-deficient knockout mice.} To investigate the physiological function of \textit{Malat1} and to study the consequences of \textit{Malat1} deficiency in vivo, we established an inducible \textit{Malat1} knockout mouse model by homologous recombination in murine embryonic stem (ES) cells. The complete 6,982 bp long \textit{Malat1} sequence was deleted, including 250 nucleotides upstream of the transcriptional start site and 321 nucleotides following the 3’-end of the \textit{Malat1} transcript. Detailed information on the production of the knockout mice is provided in the Materials and Methods section. A schematic presentation of the targeted \textit{Malat1} locus, its construction by homologous recombination in ES cells as well as Cre- and Flp-mediated deletions in mice are shown in Figure 4A. Verification of correct \textit{Malat1} targeting in ES cells and mice were performed by PCR and Southern Blot analysis (Fig. 4B and C).

For our initial analysis, we generated mice with a constitutive knockout of \textit{Malat1} by mating of animals harboring one conditional knockout allele with transgenic CMV-Cre deleter mice. The Cre-mediated deletion of \textit{Malat1} was confirmed by PCR analysis (Fig. 4D). The resulting heterozygous knockout mice were bred with each other to obtain \textit{Malat1}-deficient offspring. Surprisingly, homozygous \textit{Malat1}\textsuperscript{-/-} mice were born alive, without indication of embryonic lethality (in total, 15 homozygous \textit{Malat1}\textsuperscript{-/-} knockout mice, 21 heterozygous \textit{Malat1}\textsuperscript{-/+} knockout animals and 7 wild-type \textit{Malat1}\textsuperscript{+/+} mice were obtained), and these animals displayed no apparent phenotype or pathological limitations when kept under normal stress-free conditions.

\textit{Malat1} and \textit{Neat1} expression after knockout of \textit{Malat1}. To validate the effective knockout of \textit{Malat1} in the mouse model, we determined the expression of \textit{Malat1} transcripts in eight different organs by qRT-PCR (Fig. 5A). As expected, \textit{Malat1} expression was absent in all tissues obtained from homozygous \textit{Malat1} knockout mice. Importantly, we used three different amplicons covering the entire length of

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**Figure 4.** Generation and confirmation of the \textit{Malat1} knockout mouse line. (A) Schematic presentation of the strategy for deletion of \textit{Malat1} by homologous recombination in the mouse genome. \textit{LoxP} (Cre recombination) and \textit{FRT} (Flip recombination) sites are indicated and allow individual removal of the complete \textit{Malat1} locus (Cre) and the Neomycin (neo) resistance gene (Flip), respectively. Probe 2: 3’- external probe (located outside of the targeting vector) for Southern blot analysis. (B) PCR analysis of genomic DNA prepared from single electroporated ES cell clones. With the forward primer located in the neo resistance gene and the reverse primer hybridizing to the wild-type sequence at the 3’-end outside of the targeting construct, several ES cell clones with the desired homozygous recombination were identified via a 2,420 bp PCR fragment which is absent in clones without correct targeting. (C) Southern Blot analysis with genomic DNA prepared from single electroporated ES cell clones and digested with the restriction enzymes BamHI and XhoI. Probe 2 recognized a 4.1 kb XhoI/BamHI fragment in the correctly targeted locus, while the wild-type allele was identified via a 10.6 kb BamHI/BamHI fragment. All relevant restriction sites are indicated in (A). (D) To confirm Cre-mediated deletion of \textit{Malat1} and to distinguish wild-type, heterozygous and homozygous \textit{Malat1} constitutive knockout mice, a three-primer-PCR-strategy was developed, resulting in two fragments with different lengths for the wild-type (120 bp) and the targeted (204 bp) locus.
Malat1 to validate the quantitative loss of Malat1 over the entire transcript. Additionally, the expression of the lncRNA Neat1 was determined using two independent amplicons. Neat1 is a nuclear lncRNA that is essential for paraspeckle formation and represents the neighboring transcript upstream of Malat1. In only four out of eight organs, we detected a slight, but non-significant induction of Neat1 in Malat1-negative tissues arguing against a hypothetical role of Malat1 in regulating Neat1 expression in cis.

In the human MALAT1-deficient cell lines, NEAT1 expression also remained unaltered (Fig. 5B).

Histomorphological analysis of WT and Malat1 KO organs. Different organs of Malat1 KO mice were analyzed histomorphologically, including brain, heart, lung, thymus, liver, spleen, pancreas, kidney, intestine and genitals (ovary and testis). Notably, we did not observe any differences related to organ development, organ architecture or organ size. Moreover, no tumors or signs of inflammation were detected in any of the analyzed tissues. Representative pictures of two wild-type (♀, ♂; Fig. 6, left panel) and two knockout mice (♀, ♂; Fig. 6, right panel) of brain, thymus, lung, liver, pancreas, kidney and genitals are shown.

Brain sections show proper assembly of the brain cortex with pyramidal neurons and oligodendroglial cells (Fig. 6A). Thymic parenchyma is regularly divided into a cortical portion with densely packed lymphoid cells and a pale stained medullary portion (Fig. 6B). Lung tissue showed a regular alveolar structure both in WT and Malat1 KO mice without any signs of edema, congestion or emphysema (Fig. 6C). Liver parenchyma displayed the characteristic lobular organization with hexagonal appearance based on the distributions of portal areas and central venules. No signs of fibrosis or inflammation were visible (Fig. 6D). Pancreatic tissue of both wild-type and Malat1 knockout mice was regularly subdivided into the exocrine part composed of acini and the pale stained endocrine part with prominent islets of Langerhans (Fig. 6E). Mouse kidneys of WT and Malat1 KO mice showed a regular assembly of renal cortex showing glomeruli and tubules (Fig. 6F) as well as regularly structured medulla with loops of

Figure 5. Malat1 and Neat1 expression. Malat1 and Neat1 expression were determined in eight different mouse tissues (A) and two human cancer cell lines (B) by qRT-PCR. (A) Malat1 was quantified with three independent amplicons and Neat1 was quantified using two independent amplicons in lung, spleen, small intestine, kidney, brain, colon, liver and heart of four wild-type (WT) and three Malat1 knockout mice (KO). Gapdh served as reference gene and data was normalized to expression in normal lung which displayed the highest Malat1 and Neat1 expression. Depicted is the average expression +SEM (B) NEAT1 was quantified in A549 and HLE cancer cell lines in the parental cell line, two wild-type clones (WT) and three MALAT1 knockout clones (KO) each. RN7SL1 served as reference gene, and data was normalized to expression in the wild-type cells. Depicted is the average expression +SEM.
Loss-of-function models were successfully established in human cancer cell lines as well as in a knockout mouse model. The zinc finger nuclease-mediated insertion of polyA signals into the MALAT1 locus has been proven to be effective in gene silencing in lung cancer cells before. Here, this methodology also effectively shuts down MALAT1 expression more than 200-fold in human liver cancer cells. Hence, this knockout approach is more effective and more specific than standard RNA interference techniques to deplete this highly abundant lncRNA. In addition, we have generated Malat1 knockout mice using homologous recombination in ES cells to study the physiological function of Malat1 in a whole mammalian organism.

Remarkably, we find MALAT1 to be dispensable for cell viability, proliferation and development in the human and the mouse system, respectively. Given its many reported functional associations, its high expression, regulation and evolutionary conservation, this result is very surprising—in particular considering three previous findings: First, MALAT1 knockdown via RNA interference suppressed proliferation in CaSki cervical cancer cells unraveling a critical role for MALAT1 in this cancer hallmark capability. Second, MALAT1 had been found to govern the transcription of growth control genes. Lastly, MALAT1 had been associated with the trophoblast invasion and placenta function. Despite all of the above mentioned evidence pointing to an important and potentially essential function of MALAT1, our results clearly demonstrate that its quantitative loss in cancer cells does not affect proliferation and its knockout in mice does not affect viability or normal development.

These apparent discrepancies lead to the hypothesis that MALAT1 could have cell type-specific functions, e.g., might be important for cervical cancer cell proliferation, but not for other cell types including A549 and HLE cells. Depending on further in-depth investigations, it might serve as a paradigm for a lncRNA that is ubiquitously expressed and highly conserved but nevertheless fulfills multiple different, non-essential and tissue-specific functions depending on the cellular environment or the availability of RNA-binding interaction partners.

Our findings also emphasize the need for clean and quantitative genetic loss-of-function models—as exemplified here in both a human as well as a murine system. RNA interference-based knockdown using siRNA or shRNA constructs undisputedly has its value in interrogating a gene for its function in vivo, but these approaches also need to be critically questioned for the efficiency and specificity of their knockdown, for possible off-target effects, and they need to be reproduced with a large number of siRNAs or shRNAs to validate a gene-specific effect. However, definitive answers on the question whether a gene is essential for metazoan development and viability or not can only be derived from knockout models.

While the Malat1 knockout mouse does not display any detectable developmental or lethal phenotype, future studies will be essential to expose these mice to different stresses and environments. Such experiments will potentially reveal differences between wild-type and knockout animals that are due to the loss of MALAT1 and further uncover its function in health and disease. Most interesting will be the studies on potential

**Discussion**

The highly abundant, nuclear and evolutionarily conserved lncRNA MALAT1 had been associated with many important cellular functions, linked to molecular mechanisms and was found to be deregulated in many physiological and pathological conditions. Thus, we aimed to generate genetic knockout models to study its function and significance in vivo.

Figure 6. Depletion of Malat1 does not alter organ histomorphology. H&E staining of brain (A), thymus (B), lung (C), liver (D), pancreas (E), kidney (F) and genitals (G) from wild-type Malat1+/+ (left panel; ♀, ♂) and knockout Malat1−/− (right panel; ♀, ♂) mice. No histomorphological differences were observed between wild-type and knockout mice in all tissues examined.
tumor phenotypes by crossing these mice to different cancer models since MALAT1 has been discovered and most often linked to malignant diseases. The first description of MALAT1 had identified it as a marker in lung cancer metastasis and the human and murine knockout models presented here allow the in-depth analysis whether MALAT1 might also represent an active player in the metastatic cascade despite not being an essential gene for life and development.

**Materials and Methods**

**Cell culture.** A549 lung adenocarcinoma cells were purchased from ATCC (CCL-185). HLE hepatocellular carcinoma cells were a kind gift of Dr Britta Skawran (Hannover Medical School). Cells were cultured at 37°C, 5% CO2 in DMEM + 10% FBS, 0.2 mM Glutamine and antibiotics. A549 and HLE MALAT1 KO cells were generated as previously published.41

**RNA isolation and DNase I digest.** RNA from cancer cell lines was isolated using the TRIzol reagent (Life Technologies) according to the manufacturer’s recommendations. Samples were treated with DNase I (Roche) for 30 min at 37°C followed by a phenol:chloroform extraction and an ethanol precipitation at -80°C. For mouse tissue RNA isolation from snap frozen samples, the AllPrep RNA/DNA kit (Qiagen) was used according to the manufacturer’s recommendations.

**Reverse transcription and qRT-PCR.** RNA (1–2 μg) was reverse transcribed with RevertAid™ reverse transcriptase (Thermo Scientific) according to the manufacturer’s recommendations. Complete removal of genomic DNA was controlled in minus-RT samples in which the reverse transcriptase was replaced by water. For qRT-PCR, the ABI PowerSYBR Green PCR Master Mix was used, and the analysis was performed with an ABI StepOne Plus cycler (Life Technologies). Primer sequences can be found in Table 1.

**Proliferation assay.** Cell proliferation was analyzed with the Cell Proliferation ELISA BrdU assay (Roche) according to the manufacturer’s recommendations. Briefly, 5 × 103 were seeded into black 96-well plates with a clear bottom (Greiner Bio-One). After 48 h, the BrdU solution (10 μM f.c.) was added and the cells were incubated for 6 h. The chemiluminescence was measured with a luminometer (Fluoroskan Ascent FL; Thermo Scientific).

**Cell cycle analysis.** Cell cycle distribution of exponentially growing cells was analyzed according to standard protocols using Propidium Iodide (PI) staining for DNA content. Briefly, 3 × 105 cells were seeded 48 h prior to analysis in 10 cm dishes in normal growth medium. Cells were washed twice in phosphate buffered saline (PBS), scraped off the plates using a rubber policeman, fixed in ethanol, and RNA was digested with RNase A. Cell pellets were resuspended in PI staining solution (40 μg/mL f.c. PI in PBS). Cell cycle profiles were acquired using a BD FACS Calibur and analyzed using the Cell Quest Pro software (Becton Dickinson).

**Fluorescence microscopy analysis of nuclear structures.** Immunofluorescence, RNA staining via labeling of incorporated ethynyl uridine and staining of the DNA with 4’,6-diamidino-2-phenylindol-dihydrochlorid (DAPI) were conducted after para-Formaldehyde (PFA) fixation as described previously.43 The antibodies used detected active RNA polymerase II (H5, GeneTex), lamin A (provided by Harald Herrmann-Lerdon, DKFZ), UBF (provided by Ingrid Grummt, DKFZ), SC35 (mouse α-SC35, Sigma-Aldrich), HP1α (Euromex) and TRF2 (Calbiochem/Merk). For confocal fluorescence imaging, a Leica TCS SP5 confocal laser scanning microscope equipped with a HCX PL APO lambda blue 63×/1.4 NA oil immersion objective or HCX PL APO lambda blue 63×/1.4 NA oil immersion objective.

**Table 1. Primers used for qRT-PCR analysis**

| Gene       | Forward               | Reverse               |
|------------|-----------------------|-----------------------|
| human MALAT1 5’end | GAA TTG CGT CAT TTA AAG CCT AGT T | GTT TCA TCC TAC CAC TCC CAA TTA AT |
| human MALAT1 3’End | AAA GCA AGG TCT CCC CAC AAG | GGG CTG TGC TAG ATC AAA AGG CA |
| human NEAT1 | CCA GTT TTC CGA GAA CCA AA | ATG CTG ATC TG TGC GTA TG |
| murine Malat1 Amplicon 1 | TGA AAA AGG AAA TGA GGA AAA AAG | CCT CAC AAA ACC TTCTT T AAT |
| murine Malat1 Amplicon 2 | TCC CAA AAA GAC CTG TAG AGC TG | AGG AAT TTT TAA GAG GCT GGA TG |
| murine Malat1 Amplicon 3 | TTT TCC CCT TGC CTG TAA TTT | CAC CCC AAC AAC TTCTAC AA |
| murine NeuT Amplicon 1 | GTG GTT TGA TGG GAA TAA CAG T | GCT CCT CCC CTT GTG GTA A TTT T |
| murine NeuT Amplicon 2 | AGA AGA TTG CTT AAG GTG TAG GAC | TTT CAG TTA AGA ATC CCT TG ACC |
| human RN7SL1 | ATC GGG TGT CCG CAC TAA GTT | CAG CAC GGG AGT TTG CAC CT |
| human RPLP0 | GGC GAC CTG GAA GTC CAA CT | CCA TCA GCA CCA CAG CCT TC |
| murine Gapdh | TGG TGA AGC AGG CAT CTG AG | TGC TGT TGA AGT CGC AGG AG |

All sequences are given in the 5’–3’ orientation. The antibodies used detected active RNA polymerase II (H5, GeneTex), lamin A (provided by Harald Herrmann-Lerdon, DKFZ), UBF (provided by Ingrid Grummt, DKFZ), SC35 (mouse α-SC35, Sigma-Aldrich), HP1α (Euromex) and TRF2 (Calbiochem/Merk). For confocal fluorescence imaging, a Leica TCS SP5 confocal laser scanning microscope equipped with a HCX PL APO lambda blue 63×/1.4 NA oil immersion objective was used.

**Establishment of an inducible Malat1 knockout mouse model.** The mouse Malat1 gene locus was subcloned from the BAC clone RP24-290K10 (BACPAC Resources Center, Children’s Hospital Oakland Research Institute; the library RP24 was generated from a single male C57Bl/6J mouse). For the construction of the Malat1 knockout targeting vector, a previously published methodology and protocol was adjusted and used. Briefly, short 500 bp homology arms (one 7 kb upstream of the Malat1 transcription start (5’-arm) and one 1.9 kb downstream of the Malat1 transcript (3’-arm) were ampliﬁed by PCR with BAC clone RP24_290K10 as template DNA and inserted via restriction/ligation into the pKoII vector backbone. The BAC clone was electroporated into the recombination-competent E. coli strain SW106. The bacteria are available from the NCI Frederick (https://notendur.hi.is/bmo/Recombineering20 Website.htm). The bacteria were transformed with the Xhol/SalI linearized pKoII vector. In the bacteria, the Malat1 locus with the
7 kb upstream and the 1.9 kb downstream areas was recombined into the pKOI vector backbone. Genomic sequences directly downstream of the murine Malat1 gene were amplified by PCR and inserted in front and behind the FRT-Neo-FRT-loxP cassette in the pI45.1 vector. The 5'-arm-FRT-Neo-FRT-loxP-3'arm fragment was excised by KpnI, SacII and ApaLI and recombined downstream of the Malat1 gene into the pKOI vector. Restriction by SpeI and ligation of the loxP double-stranded oligonucleotide 5' of the Malat1 transcription start in the pKOI vector completed the Malat1 knockout targeting construct which was verified by sequencing.

The (5'-arm-loxP-Malat1-FRT-Neo-FRT-loxP-3'-arm) targeting vector was linearized by NotI and electroporated into the embryonic stem cell (ES) line V6.5 (SV129 x B6 F1 hybrid). G418-resistant ES clones were screened for correct homologous recombination at the Malat1 locus via colony PCR and Southern Blot analysis. For the colony PCR reaction, the following primer sequences were used: neo forward 5'TGA GGG GAT CAA TTC TCT AGA GCT CGC-3' (located in the neo gene sequence) and wt reverse 5'CTC ACC TGG AAC CCT CTA GTG TG-3' (located immediately downstream and outside of the targeting vector within the wildtype sequence). In case of correct homologous recombination of the targeting vector, a 2,420 bp PCR fragment was amplified (Fig. 4B). For Southern Blot analysis, the external probe 2 was generated by PCR amplification of genomic sequence using the primers fwd_probe2 (5'-AGC TCT GAG TGC CTG TTT CTG-3') and rev_probe2 (5'-ATG CTC TCT CCC ACA TGA CC-3'). The resulting 680 bp fragment was subcloned and used for conventional Southern Blotting using established protocols. Genomic DNA isolated from ES cell clones was digested with BamHI and XhoI; hybridization with the [32P]-labeled probe 2 produced a 10.6 kb BamHI/BamHI wildtype signal and a 4.1 kb XhoI/XbaI signal in case of correct targeting of the Malat1 locus (Fig. 4C).

Positive ES cell clones were injected into blastocysts derived from C57Bl/6j mice (Charles River), and chimeric animals were set up for breeding with wildtype C57Bl/6j mice to identify animals transmitting the correctly targeted Malat1 locus via the germ line. The heterozygous Malat1loxP,FRT-Neo/+ mice generated from the ES cell clones were crossed with a transgenic CMV- Cre deleter line (in a C57Bl/6j genetic background) to remove the PGK-Neo selection cassette and the complete Malat1 gene, which was confirmed by PCR (Fig. 4D and below). As a result, nucleotides 579570-5802920 from mouse chromosome 19qA (Assembly NCBI37/mm9) consisting of the complete 6982 nt Malat1 transcript sequence plus 251 nt upstream of the Malat1 transcription start site and 322 nt downstream of the Malat1 transcript end were removed.

Genotyping of constitutive Malat1 knockout mice. A three-primer-PCR strategy was used for genotyping the constitutive Malat1 knockout mice. Primer 1 (5'-CAC TCT GGG AAT GTT TTT GG-3'), Primer 2 (5'-CAG GAA AAC GCA AAA GGT GT-3') and Primer 3 (5'-TGT CGA AAA GAG GTG GTG TG-3') as indicated in Figure 4D (10 μM each) produced a 120 bp fragment for the wild-type allele and a 204 bp fragment for the Malat1-deleted locus. PCR conditions were as follows: 4 min 95°C, followed by 30 cycles of 30 sec 95°C, 30 sec 56°C, 30 sec 72°C, and a final elongation for 10 min at 72°C.

Histomorphological analysis. Mice were sacrificed at the age of 6 weeks. Tissues were resected, fixed in 4% formaldehyde and embedded in paraffin for subsequent staining. Five micrometer sections were cut and stained with hematoxylin and eosin (H&E) according to standard procedures. Histopathologic review of the H&E slides for histopathologic abnormalities was performed using an Olympus BX51 microscope and pictures were taken using a ProgRes® CMOS camera (Jenoptik). All animal experiments were done in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and according to the regulations issued by the Committee for Animal Rights Protection of the States of Hessen (Regierungspräsidium Darmstadt).

Statistical analysis. Statistical analyses were performed using Excel 2007. Significance was assessed using T-Tests after determination of the variance equality using an F-Test.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Note Added in Proof
While our manuscript was in press, two novel publications came to our attention describing similar phenotypes for the Malat1 knockout mice from the laboratories of David Spector and Shinichi Nakagawa and Kannanganattu Prasanth.
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