Expression and functions of long non-coding RNA *NEAT1* and isoforms in breast cancer

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Abstract

NEAT1 is a highly abundant nuclear architectural long non-coding RNA. There are two overlapping *NEAT1* isoforms, *NEAT1_1* and *NEAT1_2*, of which the latter is an essential scaffold for the assembly of a class of nuclear ribonucleoprotein bodies called paraspeckles. Paraspeckle formation is elevated by a wide variety of cellular stressors and in certain developmental processes, either through transcriptional upregulation of the *NEAT1* gene or through a switch from *NEAT1_1* to *NEAT1_2* isoform production. In such conditions, paraspeckles modulate cellular processes by sequestering proteins or RNA molecules. *NEAT1* is abnormally expressed in many cancers and a growing body of evidence suggests that in many cases, high *NEAT1* levels are associated with therapy resistance and poor clinical outcome. Here, we review the current knowledge of *NEAT1* expression and functions in breast cancer, highlighting its established role in postnatal mammary gland development. We will discuss possible isoform-specific roles of *NEAT1_1* and *NEAT1_2* in different breast cancer subtypes, which critically needs to be considered when studying *NEAT1* and breast cancer.

Background

Long non-coding RNAs (lncRNAs) are a diverse group of regulatory non-protein-coding transcripts defined by their size as being longer than 200 nucleotides and by their lack of long open reading frames.[1-4] Although lncRNAs display different functions and mechanisms of action, many of them seem to regulate gene expression at either transcriptional or post-transcriptional levels. LncRNAs are versatile molecules that can exert their functions by interacting with DNA or other RNA molecules via complementary base pairing, or with proteins by adapting specific secondary structures. Generally, functional mechanistic studies of lncRNAs have been severely limited due to their general low expression levels and high tissue specificity. In this regard, nuclear paraspeckle assembly transcript 1 (*NEAT1*) stands out as an exception, being a highly abundant structural RNA in the nucleus of mammalian cells with assigned functions in developmental processes and cellular stress responses.[5-7] Importantly, abnormal *NEAT1* expression is associated with serious diseases such as neurodegenerative disorders and cancer.[8-11]

There are two overlapping isoforms of *NEAT1*, *NEAT1_1* and *NEAT1_2*, which display strikingly different features in terms of their biogenesis and processing, sub-nuclear localization, and expression pattern.[6] The cellular roles of *NEAT1* in both normal and pathological conditions have largely been ascribed to the essential function of *NEAT1_2* in the assembly of a class of nuclear ribonucleoprotein

(RNP) bodies called paraspeckles.[5-7] Paraspeckles are dynamic structures that regulate cellular processes by sequestering specific proteins and RNA molecules. *NEAT1* expression and paraspeckle formation are upregulated by cellular stress and at certain developmental stages.[5-7] *NEAT1* is abnormally expressed in many breast cancers, which might partially reflect its established role in postnatal development of the mammary gland. Here, we summarize the current knowledge within this field. We argue why it in future studies is important to address potential isoform-specific functions of *NEAT1_1* and *NEAT1_2* in both normal and pathological conditions. In this review, we will use *NEAT1* when we refer to the gene, both RNA isoforms, and in contexts where the *NEAT1* isoforms are not specified.

NEAT1 and paraspeckles

Two isoforms of *NEAT1* are generated by alternative transcriptional termination and processing

Human *NEAT1* was identified along with its murine orthologue (*Neat1*) by Hutchinson *et al.* in 2007.[12] As opposed to many other lncRNAs, *NEAT1* is conserved across mammals.[12,13] Human *NEAT1* is transcribed from the multiple endocrine neoplasia (MEN) type 1 locus on chromosome 11q13 into two overlapping monoexonic transcripts: *NEAT1_1* of 3.7kb and *NEAT1_2* of 22.7kb (Fig.1).[14-16] The two isoforms share a common promoter but are generated by alternative transcriptional termination and processing at their 3' ends. *NEAT1_1*, which completely overlaps with the 5´ end of *NEAT1_2*, is generated when transcription is terminated by a polyadenylation signal (PAS), and the transcript is canonically processed by 3' polyadenylation.[17] Experimental evidence suggests that binding of the NUDT21/CPSF5-CPSF6 complex, known as the Cleavage factor Im (CFIm), to UGUA sites upstream of the PAS, directs cleavage and 3' polyadenylation of *NEAT1_1*.[17] The *NEAT1_2* isoform forms when heterogeneous nuclear ribonucleoprotein K (HNRNPK) binds to the primary transcript and suppresses the CFIm complex by sequestering the NUDT21/CPSF5 protein.[17] The PAS is consequently ignored, allowing for continued transcription and formation of *NEAT1_2*. Recently, it was demonstrated that the Integrator complex also contributes to the regulation of the relative abundance of *NEAT1_1* and *NEAT1_2*.[18] The Integrator complex associates with the C-terminal domain (CTD) of RNA polymerase II and regulates transcriptional termination and processing of a range of both non-coding and protein-coding transcripts.[19,20] Several subunits of the Integrator complex, including the catalytic IntS11 endonuclease, bind to the 5' region of *NEAT1* and cleave off and facilitate the maturation of the *NEAT1_1* transcript. This reduces the *NEAT1_2* levels in the cells. A *NEAT1_2* suppressive function has also been demonstrated for the paraspeckle-associated protein TDP-43 that enhances the *NEAT1_1* PAS activity, and consequently *NEAT1_1* formation, in pluripotent embryonic stem cells (ESCs).[21] When the ESCs are experimentally induced to differentiate into either of several alternative lineages, TDP-43

levels drop and the synthesis of *NEAT1_2* increases. In contrast to *NEAT1_1*, the long *NEAT1_2* isoform is not polyadenlyated, but processed by RNAse P cleavage and subsequently stabilized through formation of a triple helical structure at its 3' end (Fig. 1).[22,23]

_NEAT1_2_ is essential for paraspeckle assembly

NEAT1_2 is essential for the formation of paraspeckles, dynamic nuclear RNP bodies that phase-separate from the nucleoplasm to form liquid drop-like structures commonly referred to as membrane-less organelles (Fig 1).[15,16,24-30] More than 60 proteins have been reported to localize to paraspeckles, of which the Drosophila behaviour/human splicing (DBHS) family members NONO, SFPQ, and PSPC1 are the most well-described.[5,17,31-38] NONO and SFPQ, along with HNRNPH3, HNRNPK, DAZAP1, FUS, and RBM14, are essential for the formation of paraspeckles (Fig 1).[17] The majority of the paraspeckle-associated proteins contain one or more conserved RNA binding domains such as the RNA recognition motif (RRM), and have multiple functions in RNA metabolism like transcription, splicing, and nucleocytoplasmic transport.[17,26,36] Paraspeckles, like many other RNP bodies, assemble through a phenomenon called liquid-liquid phase separation (LLPS), and appear as dense liquid droplets.[25,26,39] LLPS is primarily driven by the presence of a class of low complexity domains called prion-like domains (PrLD) that are present in many paraspeckle proteins including FUS and RBM14.[25,26,40,41] In general, paraspeckles are highly dynamic structures that increase in number and size, and often change morphology to become more elongated, in response to extracellular cues that elevate *NEAT1_2* expression.[27,42-44] The structure and highly ordered organization of paraspeckles have been well described and have been the subject of some excellent recent reviews.[5-7,45]

In contrast to *NEAT1_2*, *NEAT1_1* expression is not sufficient to induce paraspeckle formation and RNA fluorescent *in situ* hybridization (RNA-FISH) analyses using probes that either recognise both isoforms or sorely the *NEAT1_2*, clearly suggests that *NEAT1_1* can localize to structures that are distinct from paraspeckles referred to as microspeckles (Fig. 1).[16,46,47] Generally, whereas *Neat1_1* is widely expressed in many tissues in mice, the expression pattern of *Neat1_2*, and consequently the presence of paraspeckles, are more restricted, being most pronounced in the surface epithelium of the stomach.[51] However, *NEAT1_2* expression and paraspeckle formation are enhanced by a wide variety of extracellular cues, and most cultivated cell lines express high levels of both isoforms of *NEAT1.*[16,24,51] Paraspeckles are absent in mouse and human ESCs, but appear when cells are induced to differentiate.[15,18,21,28,51]

NEAT1 and paraspeckles regulate cellular processes by molecular sequestration

Paraspeckles act as hubs that regulate gene expression at different levels by molecular sequestration (Fig. 2). This has been most well described for the essential paraspeckle protein SFPQ.[42,52] SFPQ is a versatile protein that regulates mRNA biogenesis at different levels, and might, depending on the promoter context, activate or repress gene transcription.[53] Elevated *NEAT1_2* levels recruit SFPQ to form paraspeckles, which lowers its concentration at gene promoters.[42,52] One example is the IL-8 encoding *CXCL8* gene that is transcriptionally activated as part of the innate immune response to viral infections.[52] Stimulation of cells with the dsRNA mimic poly I:C upregulates *NEAT1_2* expression that subsequently relocates SFPQ from the *CXCL8* promoter into paraspeckles, alleviating its repression of IL-8 expression. Recently, it was shown that elevated *NEAT1_2* levels might promote cellular differentiation by sequestering the TDP-43 protein into paraspeckles.[21] This critically interferes with the ability of TDP-43 to regulate alternative polyadenylation of a range of pluripotency-associated transcripts, including the mRNA encoding the Sox2 transcription factor. As mentioned above, TDP-43 might suppress the transcription of *NEAT1_2* by stimulating the processing of the *NEAT1_1* isoform. This indicates that there is a cross-regulation between TDP-43 and *NEAT1_2*, which contributes to cell specification.

Several reports have demonstrated that *NEAT1* and paraspeckles can regulate gene expression by retaining certain mRNAs in the nucleus, preventing their export to the cytoplasm and consequently their translation into proteins (Fig. 2).[28,43,54,55] This is most well-described for a group of frequently retained mRNAs that form double-stranded RNA hairpins in their 3´ untranslated regions due to the presence of inverted repeated Alu elements (IRAlus).[28] Nuclear retention of IRAlus-containing mRNA is mediated by their direct interaction with NONO and is dependent on *NEAT1* expression and paraspeckles. Interestingly, it was recently reported that the formation of paraspeckles in pituitary cells followed a circadian rhythm, causing rhythmical retention of a range of IRAlus-containing mRNAs.[54] Moreover, upon mitochondrial stress and dysfunction, many nuclear-encoded mitochondrial mRNAs are retained in paraspeckles, implicating that *NEAT1* and paraspeckles can regulate mitochondrial biogenesis.[43]

It has also been demonstrated that *NEAT1_2* and paraspeckles facilitate the processing of pri-miRNAs to pre-miRNAs by acting as a platform that brings together pri-miRNAs and the Drosha/DGRC8 microprocessor (Fig. 2).[56] This suggests that increased *NEAT1_2* expression is associated with a general increase in the amounts of mature miRNAs. On the other hand, *NEAT1* has been suggested by many research groups to function as a competing endogenous RNA that regulate gene expression by sponging miRNAs (recently reviewed in the context of cancer in [10]). However, cellular experiments

validating this mechanism that properly describe how a nuclear transcript like *NEAT1* can sponge a plethora of miRNAs that primarily localize to the cytoplasm, are still lacking.

Finally, *NEAT1* has been shown to interact with and induce the formation of transcriptionally active chromatin.[48,49] For estrogen receptor alpha (ERα) target genes in prostate cancer cells, this has been suggested to be primarily mediated by *NEAT1_1* in a *NEAT1_2*-independent fashion.[48] However, several lines of evidence indicates that there is an intimate crosstalk between paraspeckles and chromatin, supporting the notion that paraspeckles represent a class of nuclear condensates that might affect RNA metabolism by chromatin interaction (reviewed in [58]).

***NEAT1* and paraspeckles are upregulated by cellular stress**

Soon after its discovery, it was found that the *NEAT1* transcript and paraspeckle formation were upregulated in cells that were induced to differentiate.[15,28,59] It was also noted that *NEAT1* was identical to a previously discovered lncRNA referred to as VINC (virus-inducible ncRNA) that was found to be upregulated in mice infected with Japanese encephalitis virus or Rabies virus.[60] *NEAT1* expression is indeed induced upon infection by a range of RNA- and DNA viruses and suggested to have a role in the innate immune system.[52,61-65] In a search for conditions that influence paraspeckle formation, Hirose and co-workers found that *NEAT1* is transcriptionally upregulated when cells are exposed to proteasome inhibitors that cause formation of protein aggregates and consequently proteotoxic stress.[42] This was accompanied by formation of enlarged and elongated paraspeckles. Importantly, they showed that *NEAT1*-deficient cells were more sensitive to proteotoxic stress suggesting that *NEAT1* and paraspeckles have a role in cytoprotection. *NEAT1* was also found to be one of the most strongly upregulated lncRNA in cells that were subjected to hypoxia.[66] This is due to transcriptional upregulation of the *NEAT1* gene by HIF2α, which leads to enhanced paraspeckle formation.[67] *NEAT1* also exerts a cytoprotective role in hypoxic cells, as *NEAT1*-depleted cells are more susceptible to hypoxia-induced apoptosis than control cells.[67] Stress-induced upregulation of *NEAT1* and paraspeckles now seem to be a general theme in cellular physiology (recently reviewed in [45]), and their levels are enhanced by a series of proteotoxic, genotoxic, and metabolic stressors, as well as by agents that stimulate the innate immune system.[32,42-45,52,63,67-71] Upregulated *NEAT1* levels are primarily a result of induced transcription of the *NEAT1* gene, and a range of stress-induced transcription factors including HIF2α, HSF1, p53, ATF2, and NF-κB, activate the *NEAT1* promoter.[43,67-73]

Physiological roles of *NEAT1*

Neat1 knock out (ko) mice were first described in 2011, and even though they display compromised paraspeckle formation, they are viable and were originally reported as healthy with no distinctive phenotype.[51] This notion has now changed as several studies have been undertaken to more

thoroughly investigate their physiology and behaviour at specific physiological circumstances.[21,74-80] Most strikingly, female *Neat1* ko mice are less fertile than wild type mice and have defects in postnatal mammary gland development that critically interferes with their ability to nurture offspring.[74,75] The compromised fertility may, at least partially, be explained by impaired corpus luteum formation and reduced secretion of progesterone.[74] However, *in vitro* studies of early mouse embryos have suggested that *Neat1_2* and paraspeckles might play an important role in both preimplantation development and in the differentiation of pluripotent embryonic stem cells during gastrulation.[21,76] Thus, the reduced fertility of *Neat1* ko mice might be a consequence of severe defects in early embryonic development. Recently, it was reported that adult *Neat1* ko mice have a complex immunological phenotype with both adaptive and innate immune system alterations.[77] *Neat1* ko mice also display behavioural abnormalities that indicate defects within the central nervous system, and cultivated neurons from *Neat1* ko mice are hyper excitable and have dysregulated calcium homeostasis.[78,79]

NEAT1 is abnormally expressed in many cancers

NEAT1 is upregulated in tumour cells compared to normal cells in a wide range of human solid cancers (recently reviewed in [10,81-83]). In most cases, elevated *NEAT1* expression is associated with aggressive disease and poor clinical outcome.[8,9] *NEAT1* is upregulated in multiple myeloma, whereas *NEAT1* levels are decreased in peripheral blood or bone marrow samples from patients suffering from acute myeloid leukemia, acute lymphatic leukemia, or chronic myeloid leukemia, compared to those from healthy donors.[84-88]

Cancer whole genome analyses have detected mutational hotspots in the *NEAT1* genomic locus in many cancers, including breast cancer, prostate cancer, renal cell carcinoma, liver cancer, stomach cancer, lung adenocarcinoma, and B-cell lymphoma.[89-93] The role of these mutations in cancer development is still obscure. In general, exonic *NEAT1* point mutations do not seem to affect *NEAT1* transcript levels.[89,91] It has been suggested that exonic *NEAT1* mutants are cancer driver mutations, particularly in prostate cancer where *NEAT1* mutations were more frequently found in lethal metastases than in primary tumours.[92] This has, however, recently been questioned in a pan-cancer study of non-coding regions in 2658 cancer whole genomes where the authors argue that frequently found *NEAT1* point mutations arise from transcription-associated mutational processes, rather than through positive selection during cancer development.[90]

NEAT1 – a friend or foe in cancer?

Neat1-depletion can either suppress or enhance tumour development in mouse models. *Neat1* and paraspeckles, which are rarely found in the normal epidermis of healthy mice, are upregulated in premalignant and malignant lesions in chemical and genetic skin carcinogenesis models.[68] In a two-stage DMBA-TPA carcinogenesis model, *Neat1* ko and heterozygous mice form smaller papillomas and are less prone to develop malignant squamous cell carcinoma compared to wild type mice. This suggests that *NEAT1* might have an oncogenic role in skin cancer. *Neat1* expression is also upregulated in lung metastases compared to primary tumours in a mouse sarcoma genetic model.[94] On the other hand, *Neat1* depletion promoted pancreatic cancer initiation, and *Neat1_1* overexpression suppressed the transformation of oncogene-expressing mouse embryonic fibroblasts.[71] This indicates that *NEAT1* also can have tumour suppressor activities.

Recently, it was demonstrated that the relative abundance of *NEAT1_1* and *NEAT1_2* differs in aggressive and non-aggressive neuroblastoma cell lines.[95] Aggressive cell lines originally isolated from high-risk NMYC-amplified neuroblastoma patients, express high levels of *NEAT1_1* relative to *NEAT1_2*. In contrast, non-aggressive cells that display a more differentiated phenotype, express high levels of *NEAT1_2* and consequently have high abundance of paraspeckles. Interestingly, forced isoform switching from *NEAT1_1* to *NEAT1_2* in aggressive neuroblastoma cells, inhibited cell proliferation and induced a more differentiated cellular state. Thus, in neuroblastoma *NEAT1_1* and *NEAT1_2* seem to have opposing functions in terms of tumourigenesis, in which *NEAT1_1* acts as an oncogene and *NEAT1_2* as a tumour suppressor. As opposed to this, in ovarian cancer *NEAT1_2*-, but not *NEAT1_1* expression, is associated with reduced progression-free survival in patients treated with platinum-based therapy.[68]. Moreover, *NEAT1_2* is associated with high-grade disease in breast cancer (see below).[96] This clearly suggests that *NEAT1_2* and paraspeckles have oncogenic functions in some cancer subtypes.

NEAT1 in breast cancer

Breast cancer is a heterogeneous disease where clinical characteristics and outcome vary substantially among patients due to genomic and epi-genomic heterogeneity within the cancer cells.[97,98] Global gene expression profiling has led to the identification of intrinsic breast cancer subtypes with distinct gene expression signatures.[99,100] These signatures resemble those seen in normal breast cells at different developmental stages.[101,102] Moreover, extracellular cues regulating postnatal mammary gland development and their associated intracellular signalling proteins, are often abnormally expressed in breast cancer.[102] In clinical diagnosis, breast cancer is broadly classified into hormone receptor positive luminal cancers (ER+, PR+/-), HER2-positive cancers, and basal-like cancers that lack the expression of

hormone receptors and HER2, which are commonly referred to as triple-negative cancers.[103] An increasing number of studies have demonstrated that *NEAT1* has a role in postnatal mammary gland development and is abnormally expressed in breast cancer.

NEAT1 in postnatal mammary gland development

In mice, *Neat1* has a critical role in postnatal development of the female mammary gland. Virgin *Neat1* ko mice display defective ductal outgrowth and branching during puberty, and compromised alveologenesis during pregnancy.[75] Two days postpartum, lactating glands of *Neat1*-/- mice have fewer alveoli compared to wild type mice, critically affecting their overall milk production and ability to feed newborn pups. Consequently, the offspring of *Neat1*-/- mice has decreased survival, and reduced size and weight. Defective alveologenesis in *Neat1*-/- mice is most likely caused by decreased proliferation rate of alveolar epithelial cells at midgestation, which abrogates the expansion of the mammary epithelium that is required for the generation of a lactation-competent gland during pregnancy.[75] Importantly, although reduced in numbers, the alveolar cells of *Neat1*-/- mice express β-casein and whey acidic protein, suggesting that they still can produce milk. This indicates that even though alveologensis is severely compromised in *Neat1*-/- mice, alveolar epithelial cells still undergo lactogenic differentiation. It is worth mentioning that *Neat1* has been identified among genes that are downregulated in Stat5a knockout mice, which suggests that *NEAT1* might be a STAT5 target gene.[104,105] The JAK2-STAT5 signaling pathway is induced by prolactin, a key pregnancy hormone that is important for lactogenic differentiation and stimulates the expression of genes encoding milk proteins such as β-casein and whey acidic protein. Even though observations in *Neat1* ko mice suggest that *NEAT1* is not be involved in milk production per se, it might for instance, be required for the survival of milk producing cells.

Recently it was suggested that defective mammary gland development in *Neat1* ko mice is primarily due to lack of *Neat1_2* expression and paraspeckle formation, as pups derived from *Neat1_1* ko mice develop normally.[47] Fluorescent in-situ hybridization (FISH) analyses of wild type virgin mice have shown that *Neat1_2* and paraspeckles are present in subsets of K8/18-positive mammary luminal epithelial cells, but not in myoepithelial or stromal cells. Upon lactation, the number of *Neat1_2*-positive luminal cells increases substantially. Importantly, similar observations have been done in human mammary glands.[96] *NEAT1_2* and paraspeckles are rarely present in normal adult mammary glands. In contrast, *NEAT1_2* and paraspeckles are frequently found in luminal epithelial cells of lactating women. This indicates that *NEAT1_2* is upregulated during pregnancy and/or lactation also in humans. In line with this, *NEAT1* is reported to be among a set of genes that are upregulated in

postmenopausal samples taken from parous women compared to those taken from nulliparous women.[106,107]

NEAT1 is frequently upregulated in breast cancer

Given the role of *NEAT1* in postnatal mammary gland development, it is perhaps not surprising that *NEAT1* is abnormally expressed in breast cancer.[108] The first report on *NEAT1* and breast cancer was published in 2014 showing that *NEAT1* expression and paraspeckle formation were upregulated in hypoxic regions of breast cancer cell line xenografts.[67] Moreover, the authors reported that high *NEAT1* expression is associated with poor clinical outcome in a breast cancer cohort of ~2000 patients regardless of clinicopathological conditions like age, tumour size, stage, grade, and lymph node metastases. This suggests that *NEAT1* is an independent prognostic factor in breast cancer. To date, at least 11 studies have found *NEAT1* to be differentially expressed in tumour tissue compared to adjacent normal tissue in breast cancer patients (Table 1).[50,96,109-117] Strikingly, 10 of the 11 studies have found *NEAT1* to be more highly expressed in tumour cells than surrounding normal cells, and high *NEAT1* expression has been associated with aggressive cancer characteristics like tumour size[115], stage[116,118], grade[50,96], and lymph node or distant metastasis.[50,115,116,118]

Elevated *NEAT1* levels have also been found in plasma or peripheral blood samples from breast cancer patients compared to healthy donors.[50,119-122] Whether this is a result of active secretion by breast cancer cells, shedding and lysis of breast cancer cells, or induced expression in blood cells or other non-cancerous tissue, is currently unknown. Nevertheless, these observations might set the basis for evaluation of *NEAT1* as a potential liquid biomarker for breast cancer. In breast cancer, mutational hotspots have been found within the *NEAT1* core promoter.[91] Interestingly, the majority of these mutations are associated with decreased *NEAT1* expression *in vitro*. The consequences of these mutations on *NEAT1* expression and paraspeckle formation in-situ in tumour samples, are currently not known.

NEAT1 and paraspeckles are upregulated by malignancy-associated stress in breast cancer

Cancer cells in solid tumours generally suffer from proteotoxic, genotoxic, and metabolic stress due to hypoxia, oxidative stress, nutrient deprivation, or mutations causing hyperactivation of oncogenic pathways.[123,124] An important hallmark of cancer cells is their increased ability to adapt to and tolerate such conditions due to constitutive upregulation of cellular stress response pathways.[125-127] Increasing evidence suggests that elevated *NEAT1* expression and paraspeckle formation form part of such malignancy-associated stress response pathways (Fig. 3). In breast cancer, *NEAT1* is transcriptionally activated by HIF2α in response to low intratumoural oxygen levels.[67] *NEAT1* is also transcriptionally

upregulated by HSF1 as part of the heat shock response pathway to proteotoxic stress.[69] Constitutive nuclear localization of activated HSF1 is frequently seen in breast cancer and associated with decreased survival.[128] Thus, elevated levels of *NEAT1* in breast cancer might at least partially, be caused by increased HSF1 activity. Importantly, *NEAT1* has by several independent research groups been shown to be a bona fide p53 target gene, and consequently *NEAT1* and paraspeckles are upregulated by genotoxic stressors.[68,71,72] Although the role of *NEAT1* in p53-regulated cellular functions is still somewhat enigmatic, and conflicting results have been reported, it has been suggested that enhanced *NEAT1_2* expression and paraspeckle formation might counteract drug- and oncogene-induced DNA replication stress. By doing so, *NEAT1_2* and paraspeckles prevent accumulation of double-stranded DNA breaks and increase the tolerance to genotoxic stress.[68]

NEAT1 confers tumourigenic capabilities to breast cancer cell lines

Several research groups have reported that *NEAT1* is more highly expressed in breast cancer cell lines compared to the immortalized breast epithelial cell line MCF10A.[109,111,113,114,116,118,129,130] Experimental evidence suggests that *NEAT1* contributes to their tumourigenic capabilities as knockdown of *NEAT1* expression impairs proliferation and cell cycle progression, anchorage-independent growth, migration, and cell survival.[113-116,119,120,129-132] *NEAT1*-depletion also inhibits *in vivo* tumour formation in mouse xenograft models of MDA-MB-231 and BRCA-deficient murine mammary tumour cells.[120,132] Moreover, MCF7 cells overexpressing the *NEAT1_1* isoform has an increased propensity to metastasize to the lungs in mice.[50] Recently, *NEAT1*-deficiency was shown to decrease the stem cell-like CD44+/CD24– subpopulations of MDA-MB-231 and MCF10DCIS cells[120,132], and compromise the ability of Brca1-deficient MCF10DCIS and radioresistant MDA-MB-231 cells to form mammospheres.[132,133] Moreover, ectopic expression of *NEAT1_1* promoted mammosphere formation in MCF10A cells[132], and *NEAT1* expression was increased in 3-dimensional cultures of radioresistant MDA-MB-231 cells compared to cells cultivated in monolayers.[133] This indicates that *NEAT1* might contribute to the acquisition of cancer stem cell-phenotypes in breast cancer, which is associated drug resistance and relapse.

NEAT1 and paraspeckles confer therapy resistance to breast cancer cell lines

As mentioned above, *NEAT1_2* and paraspeckles protect cells from genotoxic stress. Importantly, this suggests that elevated *NEAT1_2* and paraspeckle levels in cancer cells might confer resistance to genotoxic drugs. It has indeed been shown that *NEAT1*-depletion leads to accumulation of double-stranded DNA breaks in MCF7 cells and enhanced sensitivity to chemotherapeutic agents such as doxorubicin, PARP-inhibitors (ABT-888), and p53 reactivation therapy (Nutlin-3a).[68] Moreover, *NEAT1* expression is elevated in MDA-MB-231 cells with acquired resistance to either cisplatin or taxol

compared to control cells, and *NEAT1* knockdown increases their sensitivity to these drugs.[120] *NEAT1*-depletion has also been found to sensitize MDA-MB-231 cells to radiation.[133]

Estrogen upregulates *NEAT1* in cell lines

NEAT1 is upregulated by 17β-estradiol (E2) in ER-positive MCF7 breast cancer as well as prostate cancer cells, and *NEAT1* can modify the expression of ERα target genes by binding to chromatin.[48-50,134] Capture hybridization analyses of RNA targets (CHART) analyses of untreated and E2-treated MCF7 cells, showed that *NEAT1* is rapidly recruited to regulatory regions of genes that are activated by E2, including the well-established ERα target gene *GREB1*.[49] *NEAT1* has also been shown to participate in a transcriptional repressor complex with SIN3A and FOXN3 in ER-positive breast cancer cells.[50] Importantly, The FOXN3-*NEAT1*-SIN3A complex was found to repress the transcription of the *GATA3* gene and *TJP1* gene encoding the tight junction protein Zonula Occludens 1 (ZO-1). Thus, emerging evidence indicates that *NEAT1* can participate in an intricate network that can affect the expression of ERα-target genes, including GATA3, either positively or negatively. Importantly, it has been suggested that it is the *NEAT1_1* isoform that regulates ERα-target genes.[48,50]

Breast cancer and distinction of *NEAT1* isoforms

The vast majority of the studies of *NEAT1* expression in breast cancer are based on RT-qPCR protocols using primers that do not distinguish between the *NEAT1_1* and *NEAT1_2* isoforms. However, a growing number of studies clearly suggest that the two isoforms have different functions both in normal cellular physiology and in cancer. There are indeed several experimental protocols allowing isoform-specific expression and functional analyses of *NEAT1*. *NEAT1_1* and *NEAT1_2* can be distinguished based on their size by traditional Northern blot analyses, but this method is cumbersome and not applicable if the number of samples is high. In both cellular models and patient samples, *NEAT1_2* expression can be specifically studied by hybridization-based technologies such as RNA-FISH and microarray analyses employing probes that solely bind to the unique *NEAT1_2* region. It is important to note that in general, care should be taken during preparation of RNA samples aimed for NEAT1_2 expression analyses as it is semi-extractable due to its association with paraspeckle proteins.[39] Moreover, RNA-sequencing of polyA-selected RNA molecules will provide specific information about *NEAT1_1* expression.[96,117]. Here, the mapping of sequencing reads should be carefully analysed to make sure that *NEAT1_2* is not pull-downed in the polyA enrichment step due to internal polyA stretches. Isoform-specific functions can also be analysed in cellular experiments where *NEAT1_2* expression is knocked down by antisense oligonucleotides (ASOs), and locked nucleic acid (LNA) GapmeRs ASOs have proven to be particularly efficient.[68,69] Moreover, *NEAT1_2* expression can

be severely compromised by CRISPR/Cas9-mediated genome editing either by insertion of a SV40-T-derived transcriptional terminator sequence downstream of the *NEAT1_1* PAS, or by deletion of regions involved in triple helix formation and stabilization of the *NEAT1_2* 3' end.[25,46] Opposingly, deletion of the *NEAT1_1* PAS by CRISPR/Cas9 severely reduces *NEAT1_1* expression and elevate *NEAT1_2* formation.[135] Recently, the same was achieved by sterically blocking the PAS by ASOs.[95,135]

Given the development of the above-mentioned protocols, isoform-specific analyses of *NEAT1* expression in breast cancer is possible. Importantly, in the pioneer work by Choudhry *et al.* demonstrating an association between high *NEAT1* expression and patient mortality in the METABRIC cohort, the authors drew their conclusion based on microarray data that only contains expression data for the *NEAT1_2* isoform.[136] In line with this, by using a *NEAT1_2*-specific RNA-FISH-based approach, Knutsen *et al.* showed that high *NEAT1_2* expression was associated with high-grade breast cancers.[96] In sharp contrast, the only study so far showing that *NEAT1* expression is higher in normal tissue than in breast cancer tissue, has analysed RNA-sequencing data of polyA-enriched transcripts from the TCGA breast cancer cohort where none of the sequencing reads mapped to the *NEAT1_2* isoform.[117] Moreover, the authors showed that high *NEAT1_1* expression was associated with good prognosis in p53 wild type breast cancers. Although further experimental evidence is required, this might indicate that high levels of *NEAT1_2*, but not *NEAT1_1*, are associated with aggressive disease in breast cancer.

Recently, it was demonstrated that *NEAT1_1* levels are regulated in a cell cycle-dependent manner.[47] Whereas *NEAT1_1* is highly expressed in resting G0 cells and throughout the G1 phase, the transcript is actively degraded by the RNA exosome when cells enter the S-phase. *NEAT1_2* levels display less fluctuation, and therefore, the *NEAT1_2* to *NEAT1_1* ratio varies throughout the cell cycle.[47] How this translate into isoform expression in hyperproliferative cancer cells, is currently not known.

NEAT1 isoform distribution varies among breast cancer subtypes

Increasing evidence suggests that the two *NEAT1* isoforms have different expression pattern among breast cancer subtypes. By performing *NEAT1_2*-specific RNA-FISH analyses of core needle biopsies, Knutsen *et al.* showed that *NEAT1_2* expression was associated with HER2-positive breast cancers.[96] This was further supported by analyses of microarray data from three breast cancer cohorts where only expression data generated from probes solely hybridizing to *NEAT1_2*- specific regions, were considered. Here, *NEAT1* _2 was found to be most highly expressed in breast cancers molecularly classified as either HER2-enriched or luminal B, which are the two intrinsic subtypes that frequently overexpress the HER2 receptor. *NEAT1_1*-specific analyses are hampered by the fact that it completely overlaps with the *NEAT1_2* transcript. However, comparison of the expression pattern of total *NEAT1*

and *NEAT1_2* has provided some hints indicating that the two isoforms display different expression patterns among breast cancer subtypes. For instance, in microarray data from the TCGA breast cancer cohort, total *NEAT1* expression as determined by probes recognizing the overlapping 5' region of *NEAT1_1* and *NEAT1_2*, shows a different distribution among the intrinsic breast cancer subtypes than *NEAT1_2*, being highest in ER-positive luminal cancers.[96] In concordance with this, by inspecting several microarray breast cancer data sets in the Oncomine database, Li *et al.* found that *NEAT1* expression is higher in ER-positive than in ER-negative cancers.[50] Although not specified in the paper, most of these data sets are generated by the Affymetrix Human Genome U133 Plus 2.0 Array that contains a mixture of *NEAT1* probes that recognize both the common and the *NEAT1_2*-specific region. Finally, analyses of polyA-enriched RNA-sequencing data from the TCGA breast cancer cohort considering 1072 (out of 1103) samples, where none of the reads mapped to the *NEAT1_2* region, showed indeed that *NEAT1_1* expression is associated with ER-positive luminal cancers.[96]

Taken together, publicly available expression data indeed suggests that *NEAT1_1* might be the dominating *NEAT1* isoform in ER-positive breast cancers as well as normal mammary epithelial cells. In contrast, a switch from the *NEAT1_1*- to the *NEAT1_2* isoform appears to be a frequent event in the generally more aggressive HER2-positive cancers. Keeping with this notion, studies should be undertaken to more thoroughly analyse the association between aggressive ER-positive luminal cancers and *NEAT1_2* expression, as this would give vital information about whether a switch from *NEAT1_1* to *NEAT1_2* is associated with advanced breast cancer in general Fig. 4 illustrates the expression pattern of *NEAT1_1* and *NEAT1_2* in the intrinsic breast cancer subtypes.

Does *NEAT1* isoform distribution reflect the cellular origin of breast cancers?

NEAT1_2 expression is associated with HER2-positive breast cancers. It is thus tempting to speculate that signaling through the ErbB receptor family stimulates isoform switching from *NEAT1_1* to *NEAT1_2*. In contrast, ER-positive cancers appear to express high levels of *NEAT1_1* suggesting that the formation of *NEAT1_2* might be actively suppressed by ER-expression. Experiments should be undertaken to determine whether the isoform expression pattern in breast cancer subtypes reflects the expression in the originating mammary epithelial cells. One might envision that ER-positive luminal cells express high levels of *NEAT1_1*, whereas for instance amphiregulin (AREG)-signaling induces isoform-switching to *NEAT1_2* in ER-negative cells. As for basal-like triple negative breast cancers, *NEAT1* expression data is somewhat conflicting. In a RT-qPCR-based study of breast cancer tissue samples, Shin *et al.* reported that total *NEAT1* expression was higher in triple negative breast cancers as compared to luminal and HER2-positive cancers, whereas no difference was found for *NEAT1_2* expression.[120] However, both Knutsen *et al.* and *Li et al.* have reported that the triple negative breast

cancer cell line MDA-MB-231, expresses low levels of total *NEAT1* and *NEAT1_2*. Moreover, in RNA-sequencing data from the TCGA cohort, lowest *NEAT1* levels are found in basal-like breast cancers. Basal-like breast cancers are poorly differentiated and thought to originate from mammary stem cells or luminal progenitor cells.[102] Even though this remains to be resolved experimentally, this might indicate that *NEAT1* expression is low in unspecified mammary cells and turned on at later stages in the mammary cell differentiation hierarchy.

Conclusion

NEAT1 and paraspeckles have over the last few years been the subject of an increasing number of studies due to their association with pathological conditions such as cancer and neurodegenerative disorders. In general, cells that are affected in such diseases are suffering from proteotoxic, genotoxic, or metabolic stress that leads to enhanced *NEAT1* expression and paraspeckle formation. Increasing evidence clearly suggests that *NEAT1* and paraspeckles have an important role in cell tolerance and survival during stress. It is thus easy to speculate that *NEAT1* levels are elevated at an early stage of disease development as a cytoprotective mechanism. For cancer management, constitutively activated cytoprotective mechanisms represent a serious challenge as they may confer resistance to therapy. *NEAT1_2* expression is indeed associated with poor prognosis in breast cancer, is a predictor of response to platinum-based chemotherapy in ovarian cancer, and *NEAT1*-depleted cells have enhanced sensitivity to chemotherapeutic drugs.[67,68] Recently, it was shown by RNA-FISH analyses that *NEAT1_2* expression and paraspeckle formation in breast cancer samples were strikingly tumour cell-specific.[96] Moreover, the fact that *NEAT1* knock out mice appear generally healthy, indicate that *NEAT1* targeting might not confer an overall toxicity problem as such. Taken together, these observations suggest that *NEAT1_2* could be a future target for cancer therapy. Recently, great progresses have been made within the field of RNA-targeting drugs based on RNA interference, antisense therapy, or small-molecule inhibitors. [137-139] Although the number of clinical studies on lncRNA targeting is still scarce being limited to antisense non-coding mitochondrial RNAs, they are regarded as attractive targets in future cancer therapy.[140] Of particular relevance to *NEAT1_2* targeting, are studies reporting the identification of small molecule inhibitors of *MALAT1* that reduced the levels of the transcript by specifically interfering with the stabilizing triple helical structure in its 3'end. [141,142] Importantly, the 3' end of *MALAT1* is structurally similar to that of *NEAT1_2* [22,23], indicating that compounds displaying similar mechanisms of action could be developed into *NEAT1_2*-targeting drugs. *NEAT1_2*-specific RNA-FISH analyses have proven to be amenable for clinical samples.[68,96] Thus, clinical studies should be undertaken to more systematically assess whether *NEAT1_2* can predict the response to neoadjuvant treatment of for instance HER2-positive breast cancers, and maybe more importantly,

test the synergy between *NEAT1*-targeting and other treatment modalities such as HER2-directed therapy.

A growing body of evidence indicates that *NEAT1_1* and *NEAT1_2* have distinct roles in cancer development, and that their relative contribution to carcinogenesis might vary between different cancer types.[68,71,95,96] This needs to be critically considered when designing protocols for studying *NEAT1* in cancer. In breast cancer, the relative expression of *NEAT1_1* and *NEAT1_2* appears to be different among the intrinsic subtypes. This might reflect distinct expression pattern of the two isoforms in different subpopulations of mammary epithelial cells. Sophisticated lineage tracing studies should be conducted to clarify the relative role of the two isoforms in postnatal mammary gland development. Moreover, it would be interesting to see whether female reproductive hormones can contribute to the regulation of *NEAT1* expression in general, and/or to isoform switching. Finally, emphasis should be put on clarifying the still enigmatic role of *NEAT1_1*, which constitutes the most evolutionarily conserved part of *NEAT1*, in cellular physiology.

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Authors' contributions

E. K., A.L.H. and M.P. conceptualized and wrote the manuscript. Correspondence to Maria Perander.

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The authors declare no competing interests.

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Table 1 Breast cancer-associated studies analysing *NEAT1* expression in tumour and normal tissue.

Fig. 1. Two isoforms of *NEAT1* with distinct functions arise from alternative transcriptional termination and processing. The human *NEAT1* gene is located within the MEN 1 locus on chromosome 11q13 and is transcribed into two overlapping isoforms, *NEAT1_1* and *NEAT1_2*. *NEAT1_1* is generated when transcription is terminated by a polyadenylation signal and is processed by 3' polyadenylation through a CFIm-dependent mechanism. TDP-43 and members of the Integrator complex stimulate the formation of *NEAT1_1*. Suppression of the polyadenylation signal by HNRNPK leads to continued transcription and formation of *NEAT1_2*, which is stabilized at its 3' end by formation of a triple helical structure. *NEAT1_2* is a scaffold for assembly of paraspeckles, nuclear ribonucleoprotein bodies that form through liquid-liquid phase separation. *NEAT1_1* localizes to paraspeckles but is not an essential component, and can also form microspeckles. The picture panel shows fluorescent confocal images of MCF7 cells subjected to *NEAT1_2*- and NONO-specific co-immuno- fluorescent in situ hybridization analyses (green and red dots, respectively). *NEAT1_2* and NONO colocalize in paraspeckles (yellow dots). Nuclei are visualized by DAPI staining (blue).

Fig. 2. Paraspeckles regulate cellular processes by molecular sequestration. *NEAT1_2* levels and paraspeckle formation are upregulated by cellular stress and in certain developmental processes. Paraspeckles regulate gene expression at transcriptional and post-transcriptional levels through protein sequestration (A) or nuclear retention of specific mRNAs (B). Although less described, paraspeckles have also been shown to facilitate pri-miRNA processing (C).

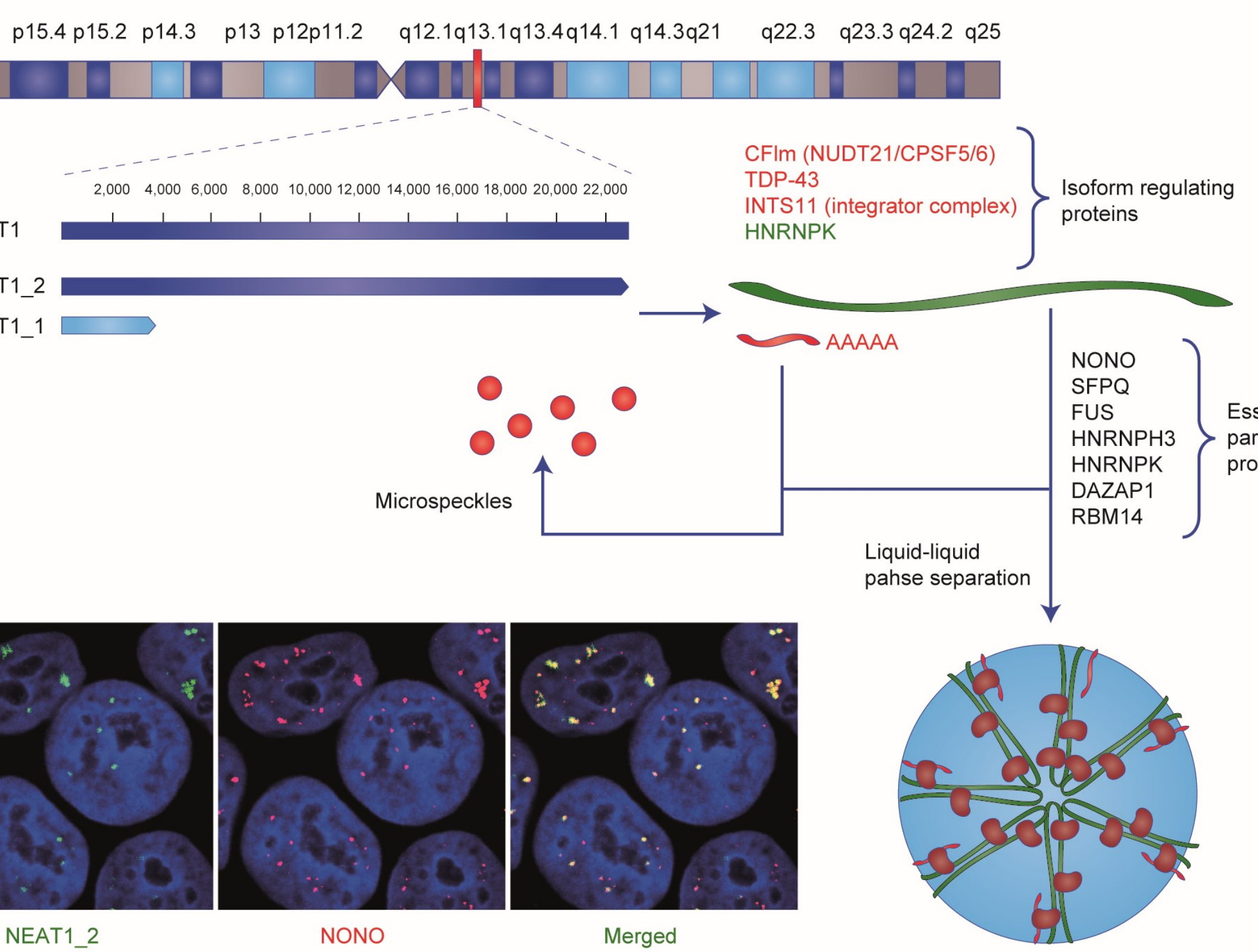
Fig. 3. *NEAT1_2* and paraspeckle formation are upregulated by malignancy-associated stress and confer therapy resistance. The *NEAT1* gene is transcriptionally upregulated by metabolic, genotoxic,

and proteotoxic stress in cancer cells. Increased *NEAT1_2* levels lead to enhanced paraspeckle formation. Paraspeckles aid cellular adaptation and tolerance to stress, which might critically affect therapy responses in cancer cells.

Fig. 4. Distinct *NEAT1* isoform distribution in breast cancer subtypes. Schematic diagram indicating *NEAT1_1* and *NEAT1_2* expression levels in intrinsic breast cancer subtypes.

Table 1 Breast cancer-associated studies analysing *NEAT1* expression in tumour and normal tissue.

Ref	N (Tum/Norm)	Method for quantification and sample type	*NEAT1 isoform*	Comment
[116]	N=70	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.
[115]	N=40	RT-qPRC/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue
[50]	N=23	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.
[117]	Unknown, not reported	RNA-Seq, TCGA/ Fresh-frozen tissue	*NEAT1_1*	T<N Higher *NEAT1_1* in normal than tumour tissue
[114]	N=43	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue
[96]	Tum=74 Norm=27	FISH/FFPE needle biopsies	*NEAT1_2*	T>N Imaging, single cell resolution: No expression of *NEAT1_2* in non-cancerous cells in patients or in healthy individuals
[113]	N=34	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.
[111]	N=40	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.
[143]	N=20	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.
[109]	Tum=23 Norm=15	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N
[110]	N=54	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T=N Adjacent normal tissue.
[112]	N=37	RT-qPCR/ Fresh-frozen tissue	*NEAT1*	T>N Adjacent normal tissue.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2,000 4,000 6,000 8,000 10,000 12,000 14,000 16,000 18,000 20,000 22,000
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T1_1
CFIm (NUDT21/CPSF5/6)
TDP-43
INTS11 (integrator complex)
HNRNPK
Isoform regulating proteins
AAAAA
NONO
SFPQ
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HNRNPH3
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NEAT1_2
NONO
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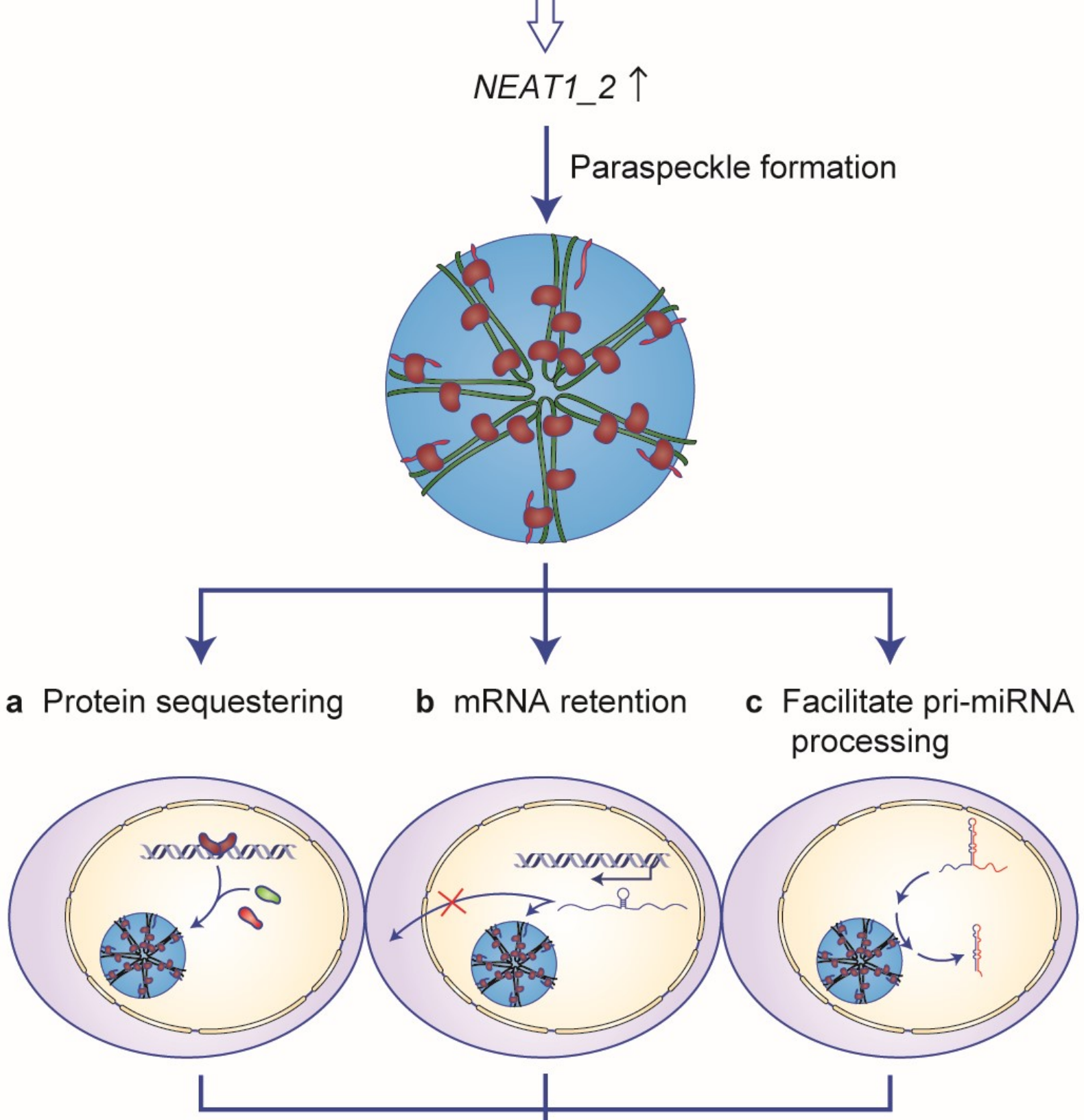
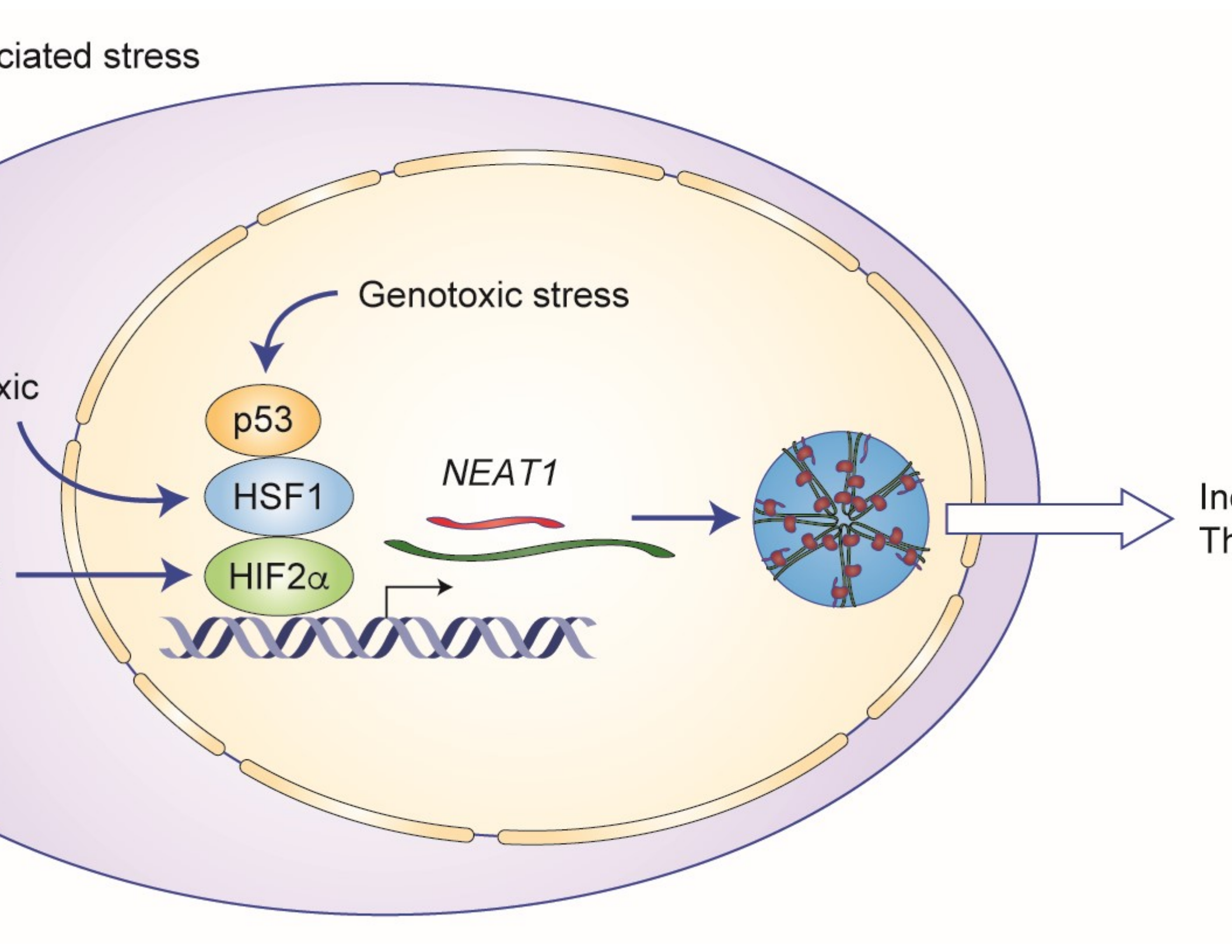

NEAT1_2 ↑
Paraspeckle formation
a Protein sequestering
b mRNA retention
c Facilitate pri-miRNA processing


ciated stress
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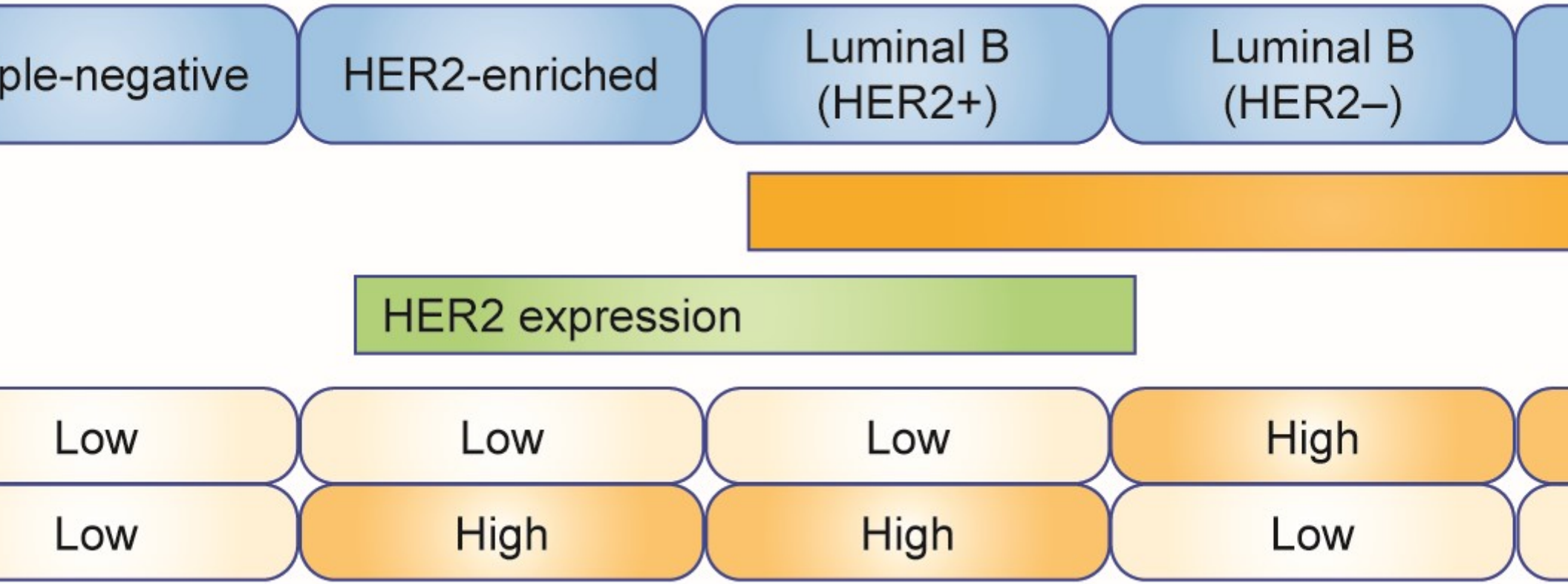
ple-negative
HER2-enriched
Luminal B (HER2+)
Luminal B (HER2–)
HER2 expression
Low
Low
Low
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Low
High
High
Low