NORAD-Regulated Signaling Pathways in Breast Cancer Progression

Subjects: Oncology

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Breast cancer (BC) is a heterogeneous disease classified into different subtypes presenting several treatment challenges, especially in more advanced cases arising from triple negative breast cancer. *NORAD* is a long non-coding RNA (lncRNA) activated by DNA damage, with an impacting role in the repair process of DNA insults. This lncRNA is differentially expressed in BC subtypes, participating in cancer initiation and progression, by interacting with an extended range of signaling partners.

Keywords: lncRNAs ; NORAD ; pumilio ; breast cancer ; chemotherapy resistance

1. PUM Proteins and Target Genes

PUM proteins are RBPs from the highly conserved Puf family. In mammals, the two canonical cytoplasmic PUM proteins are pumilio RNA binding family member 1 and 2 (PUM1 and PUM2, respectively). PUM proteins bind specifically and with great affinity to the conserved motifs of Pumilio Recognition/Response Element (PRE) found in the 3′ Untranslated Region (UTR) of their target genes, and they post-transcriptionally regulate mRNA degradation and repress protein translation [1][2]. In some cases, PUMs can act in translation to prevent their target's ubiquitination and increase protein stability [3]. Some PUM target genes, including *PARP1*, minichromosome maintenance complex component 4 (*MCM4*), the structural maintenance of chromosomes 1A (*SMC1A*) and centromere protein J (*CENPJ*), regulate important biological functions, such as DNA repair and replication, cell cycle and mitosis. *NORAD* was discovered and first described in the human colorectal cancer cell line HCT116 where in silico assays revealed repetitive sequences containing PREs, allowing for PUM1 and PUM2 binding [4]. After DNA damage induction, *NORAD* co-localizes with PUM in *NORAD*–PUM (NP) bodies in the cytoplasm where *NORAD* negatively regulates cytoplasmic PUM proteins in phase-separated condensates as ribonucleoprotein (RNP) granules. *NORAD*'s high expression and the presence of multiple PREs allows for complete and competitive PUM recruitment and the subsequent maintenance of genome stability [5]. PUM expression and its impact on BC is also being debated. Some studies report PUM1 to be one of the most differently expressed and methylated genes in BC [6] and PUM2 to have higher expression in tumors as in TNBC, where it negatively correlates with BC patient overall survival (OS) and relapse-free survival (RFS) [7]. Other studies report lower PUM2 expression in LumA and TNBC tumors compared to normal tissues and that PUM2 silencing increases cell viability, migration and invasion in cancer cells lines, while its OE produces the opposite effect [8]. Slight variations in the expression or availability of PUM proteins are sufficient to lead to CIN. In this context, the absence of *NORAD* leads to the release and hyperactivation of PUM proteins and the appearance of deleterious effects, such as accelerating premature aging in mice [9]. *NORAD* can sequester a significant fraction of PUM proteins, negatively regulating their capacity to repress target mRNAs [4]. In this line of thought, several PUM targets and their implications in BC progression will be further described below.

Ral GTPase activating protein non-catalytic subunit beta (RALGAPB) participates in the regulation of mitosis, and its dysregulation is associated with genomic instability [10]. In some cancers, such as pancreatic ductal adenocarcinoma (PDAC) and oral squamous cell carcinoma (OSCC), *RALGAPB* depletion has been reported to promote invasion, migration, tumor growth and metastasis by increasing transforming growth factor beta 1 (TGFB1) signaling and decreasing c-Jun N-terminal kinase activity [11][12] and mTORC1-dependent pancreatic tumor cell invasion [13][14]. Based on The Cancer Genome Atlas (TCGA) RNA-seq data on BC tissues and clinical data from the cBioPortal platform, PUM-binding lncRNAs were selected and evaluated in each BC subtype. Interestingly, *RALGAPB* was revealed to co-express with *NORAD* in all analyzed BC subtypes. The high expression of both *NORAD* and *RALGAPB* was associated with worse prognosis and poorer OS in LumA subtype. Moreover, both genes (combined or separately) show biomarker potential to discriminate BL and LumA from non-tumoral and BL from LumA, supporting *NORAD* as the most relevant lncRNA with PUM binding sites in BC and the molecular axis where *NORAD*, PUM and RALGAPB participate as a potential target for novel BC targeting strategies [15].

Neuropilin 1 (NRP-1) transcript and protein levels were associated with BC progression, with increased levels in BC cell lines [16], higher expression in TNBC compared to LumB [17], poorer BC prognosis [18] and higher treatment resistance [19]. In BC, *miR-376a* was reported to have decreased expression in circulation [20], tumors [21] and several cell lines, and it is positively associated with OS. Indeed, *miR-376a* OE suppressed BC cell proliferation, migration and invasion and increased apoptosis, through direct binding to *NRP-1* [22]. *PUM2* knockdown (KD) in MDA-MB-231 and MDA-MB-453 cell lines showed the attenuation of stemness properties, such as decreased expression of aldehyde dehydrogenase 1 (ALDH1) family member A1 and Nanog homeobox (NANOG) proteins, lower ALDH1 activity and decreased spheroid formation capacity. Bioinformatic analysis and luciferase assays revealed that both PUM2 and *miR-376a* bind to the 3′UTR region of *NRP-1*. Mechanistically, PUM2 and *miR-376a* compete for *NRP-1* binding, with PUM2 promoting BC stemness and *miR-376a* attenuating it. PUM2 can then induce the expression of NRP-1 by binding its mRNA and thus regulate BC progression [7].

Differential alternative polyadenylation (APA) was previously reported to be altered in BC tumors [23], and increased expression of polyadenylation components, like cleavage stimulation factor subunit 3 (*CSTF3*), was detected in TNBC cell lines. Several mRNAs with different prevalence of 3′ UTR isoforms, such as shortened and lengthened 3′UTR regions, were detected in BC tumors. It was found that PRE is the most frequently lost motif in shortened 3′UTRs in BC, but also the most often gained through APA. This suggests that PRE-containing RNAs are frequently altered by APA. Moreover, BL and TNBC tumors present more extensive and exclusive patterns of APA than LumA and LumB tumors. Gene Ontology (GO) analysis of the APA-exclusive alterations in TNBC tumors showed that the transcripts are related to the negative regulation of apoptosis, kinase activity and nucleotide binding. For instance, forkhead box O1 (*FOXO1*), a tumor suppressor transcription factor from the FOXO family group, showed extended 3′ UTR, whereas the tumor suppressor phosphatase and tensin homolog (*PTEN*), the proto-oncogene Neuroblastoma RAS viral oncogene homolog (*NRAS*) and the Jun proto-oncogene (*c-JUN*) showed recurrent 3′ UTR shortening, the latter two being the most recurring alterations. Overall, this study suggests that the dysregulated expression of *PTEN*, *NRAS*, *c-JUN* and *FOXO1* in BC relies on increased or decreased PRE-bound PUM-regulation [24], with PUM playing an important part in regulating relevant cancer-related signaling pathways.

MiR-323a-3p is a miRNA related to tumor resistance, with decreased expression in BC tissues and cell lines and tumor suppressor roles in neuroblastoma [25] and esophageal squamous cell carcinoma (ESCC) [26]. The downregulation of *miR-323a-3p* in BC cell lines results in increased viability, migration and invasion and the opposite upon *miR-323a-3p* OE. Bioinformatics and experimental assays such as RNA pulldown uncovered *NORAD* and *miR-323a-3p* binding. Moreover, *NORAD* expression directly influences *miR-323a-3p* levels, and a decrease in *miR-323a-3p* expression promotes *NORAD*-induced aggressive behavior in MDA-MB-453 cells. Bioinformatic database (Targetscan, DIANA and Starbase) analysis and RNA pulldown assays revealed that PUM1, which displays increased levels in BC tumors and cell lines, binds to *miR-323a-3p*. Indeed, *NORAD* OE impacts PUM1 expression, and *PUM1* depletion reverses the proliferation, migration and invasion capacities induced by upregulated *NORAD*, while *miR-323a-3p* negatively regulates PUM1 levels [27]. In this study, it was shown that both *NORAD* and *miR-323a-3p* can influence PUM1 and eukaryotic translation initiation factor 2 alpha kinase 3 (PERK)/eukaryotic initiating factor 2 (eIF2)/activating transcription factor 4 (ATF4) PERK/eIF2/ATF4 signaling pathway as *NORAD* OE decreases p-PERK, p-eIF2 and ATF4 protein levels. In vivo xenograft mouse models established with *NORAD*-depleted or *miR-323a-3p*-overexpressing BC cell lines reveal reduced size and weight of xenograft tumors and increased apoptosis as measured by TUNEL assay. Immunohistochemistry analysis of xenografts' tumor sections confirmed that in vivo *NORAD* inhibition results in increased *miR-323a-3p* and p-PERK and decreased PUM1 levels. In sum, *NORAD* inhibition or *miR-323a-3p* OE can decrease BC cell malignant behavior by inhibiting PUM1 and activating the downstream eIF2 signaling pathway [27].

A study using transcriptomics analysis from invasive breast carcinoma surgical tissue samples revealed the downregulation of *NORAD* in BL when compared to the LumA subtype. Survival analysis did not render any significant differences, but higher levels of *NORAD* were associated with lower DFS only in BL patients. Despite that, *NORAD* promoted accessibility, as measured using ATAC-seq, whereas methylation, from genome-wide methylation studies, was not significantly altered between the BL and LumA subtypes. Transcriptomic analysis from TCGA highlights *NORAD* as the central regulator for regulon reconstruction, revealing a network of co-expression with genes potentially modulated by *NORAD*, some of them being PUM target genes, such as the proteasome assembly chaperone 4 (*PSMG4*) [28], a proteasome assembly chaperone protein upregulated in lung neoplastic cells and correlated with poor prognosis [29]. *NORAD* regulon showed a positive activity in ER+ and PR+ tumors but was inactive in BL tumor samples. Moreover, molecular signatures and GO analysis did not reveal any significant terms between the networks of BL and LumA tumor samples, but the pathways observed were closely linked to luminal epithelial cell transformation, including BMP and ALK1 signaling. *NORAD* is thus differently expressed in BC subtypes and participates in a complex regulatory network alongside many PUM target genes [30].

Secretory carrier membrane protein 1 (*SCAMP1*) is a lncRNA that promotes cancer progression through cell viability and invasion [31]. The *SCAMP1* variant 2 (*SCAMP1-TV2*) shows increased expression in BC tumors from both LumA and TNBC subtypes and in several human BC cell lines, where *SCAMP1-TV2* silencing promotes decreased levels of PI3K and AKT, both phosphorylated and unphosphorylated forms. Evidence suggests that *SCAMP1-TV2* binds PUM2, which in turn targets INSM transcriptional repressor 1 (*INSM1*), which is able to inhibit SAM and SH3 domain containing 1 (*SASH1*), which can finally influence PI3K/AKT signaling [8]. INSM1 is a protein that regulates MYC proto-oncogene (c-Myc) and promotes BC carcinogenesis [32]. INSM1 expression is increased in human BC, and it has been proposed as a prognostic neuroendocrine marker for LumB [33][34][35]. In this study, *INSM1* OE promoted increased MDA-MB-231 and MCF-7 BC cell viability, migration and invasion and decreased apoptosis. Moreover, it reversed the BC inhibitory effects of *PUM2* OE and was accompanied by decreased expression of SASH1, a protein with tumor suppressor activity in TNBC involved in the toll-like receptor 4 (*TLR4*) signaling pathway [36][37][38][39]. Additionally, *SASH1* OE decreased BC cell viability, migration and invasion and PI3K and AKT levels, while it increased apoptosis. In vivo tumor xenograft mice models established by the inoculation of MCF-7 or MDA-MB-231 cell lines with several combinations of *SCAMP1-TV2* and PUM2 expression revealed that the simultaneous silencing of *SCAMP1-TV2* and PUM2 OE renders the highest inhibition of xenograft tumor growth [8]. PUM2 proves, yet again, its importance and broad range of targets and its ability to influence cancer-related signaling pathways.

2. NORAD-Regulated Signaling Pathways via ncRNA Sponging

There are various classes of ncRNAs, namely, transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), small RNAs (sRNAs) and lncRNAs [40]. ncRNAs can create complex networks by interacting with each other, affecting cancer cell fate and survival through different mechanisms, being considered promising diagnostic, prognostic biomarkers and therapeutic targets in cancer [41]. In particular, lncRNAs are the most predominant and diverse class among all ncRNAs [40]. They can interact with different biological molecules, such as DNA, RNA, including other ncRNAs, and proteins [41]. On the other hand, miRNAs can regulate gene expression by cleaving RNA or repressing the translation of their mRNA targets, thus regulating several biological processes such as cell cycle progression, proliferation, apoptosis and development [40]. LncRNAs can, however, act as ceRNAs by binding to miRNAs and suppress their targeting of mRNAs [42]. Next, will describe examples of ncRNAs regulated by *NORAD* with an impact on BC progression. The impact of *miR-323a-3p*, a *NORAD*-binding miRNA, was previously discussed in the context of PUM target genes (see Section 2.1).

The upregulation of *miR-155-5p* has been associated with the malignant behavior of BC cells. *MiR-155-5p* is implicated in BC by targeting suppressor of cytokine signaling 1 (*SOCS1*), a key regulator of cell proliferation and apoptosis that plays a crucial role in the degradation of ubiquitination substrates. Notably, SOCS1 acts as a tumor suppressor by facilitating the degradation of oncoproteins, inhibiting cell proliferation and apoptosis [43]. The reduced expression of *SOCS1* is linked to poor prognosis in BC patients, leading to lower OS rate as compared to high-*SOCS1*-expression patients. In the human HCC70 BC cell line, *NORAD* seems to work as a tumor suppressor through its capability to sponge *miR155-5p*, which leads to the positive regulation of *SOCS1* and a reduction in cell proliferation, migration and invasion behavior in vitro, affecting overall BC progression [44].

MiR-590-3p has been described as a tumor suppressor in several cancers [45][46][47]. In BC cells, *miR-590-3p* OE is associated with the inhibition of proliferation and higher apoptosis [45]. Moreover, *miR-590-3p* inhibits Golgi phosphoprotein 3 (GOLPH3), a protein associated with a poor prognosis and chemoresistance in BC patients [48], suggesting that *miR-590-3p* can regulate BC progression through the regulation of GOLPH3. Mechanistically, the lncRNA *NORAD* can function as a sponge to *miR-590-3p*, negatively regulating its expression and oncogenic function in the context of BC. The depletion of *NORAD* or *miR-590-3p* OE resulted in decreased MCF-7 and MDA-MB-231 BC cell proliferation, invasion and migration in vitro, with a concomitant decrease in GOLPH3 protein levels, indicating that *NORAD* might be involved in BC pathophysiology by mediating the *miR-590-3p*/GOLPH3 signaling axis [49].

A study analyzing the differently expressed transcripts between normal and TNBC, HER2+, LumA and LumB tumors predicted that *NORAD* could promote the occurrence and development of BC tumors. It proposes that *NORAD* accomplishes this by interacting with other ncRNAs like metastasis-associated lung adenocarcinoma transcript 1 (*MALAT*1) and sponging several miRNAs, including *miR-183*, *miR-182*, *miR-7*, *miR-149*, *miR-200c*, *miR-101* and *miR-342*. In turn, these miRNAs can regulate the expression of key signaling pathways, as forkhead box O3 (*FOXO3*) and ras homolog family member A (*RHOA*) [50]. The reduced expression of both *FOXO3* and *RHOA* is associated with clinical outcomes in BC, namely, metastasis, BC cell proliferation and tumorigenesis [51][52][53]. In this context, *NORAD* levels also correlate with *RHOA* and RAD51 antisense RNA 1 (*RAD51-AS1*) expression. *NORAD* is significantly increased in BC tumors compared to adjacent normal tissue, presenting a great specificity value for segregation between BC and non-tumoral tissues [54].

3. Protein- and mRNA-Mediated Regulation of Signaling Pathways by NORAD

The transforming growth factor β (TGF-β), mitogen-activated protein kinase (MAPK) and the response to DNA damage are major signaling pathways in BC. *NORAD* was shown to regulate these pathways through the differential interaction with numerous mRNA and protein partners. In particular, the *MAPK14*, a member of the MAPK family, has been described to promote BC tumor progression [55][56][57]. Although there was no significant difference in either *NORAD* or *MAPK14* levels between tumors and adjacent normal tissue, *NORAD* was shown to be significantly correlated with *MAPK14* expression in BC tumors [58].

TGF-β is a highly conserved family whose signaling is involved in different cellular processes such as cell growth, proliferation, migration and differentiation [59][60]. TGF-β signaling can either suppress or induce tumor progression, as it promotes cell cycle arrest and apoptosis in early BC stages, whereas in advanced stages, it favors cell motility, invasion and epithelial-to-mesenchymal transition (EMT) [61]. A study by Zhou et al. revealed that the upregulated expression of *NORAD* in human BC cells and patient tumors is associated with increased cell proliferation, migration and invasion in vitro and worse patient survival, by influencing the TGF-β signaling pathway. Silencing *NORAD* expression in BC cell lines leads to decreased TGF-β protein expression and the downregulation of its downstream effectors, such as SMAD family member 2 (Smad2) and RUNX family transcription factor 2 (RUNX2). In this way, *NORAD* promotes BC progression by regulating the TGF-β signaling pathway [62], highlighting the potential control of *NORAD* as a key tumor-suppressive event in BC.

In the context of BC therapy, the treatment of the TNBC MDA-MB-231 human cell line with doxorubicin triggers sustained DNA damage signals via H2A.X variant histone (H2AX) phosphorylation. Double-strand break amplification culminates in the recruitment of DNA damage signaling and repair proteins, such as BRCA1 DNA repair-associated protein (BRCA1) and tumor protein TP53 binding protein 1 (53BP1), to the damaged sites [61][63][64]. In the absence of *NORAD*, cells persist in signaling DNA damage via H2AX phosphorylation which may stem from an aberration either downstream or upstream of *NORAD*. Upon *NORAD* depletion, MDA-MB-231 cells show decreased levels of PARP1, impairing the DNA damage repair [63]. Noteworthy, PARP inhibitors are currently employed in treating advanced-stage metastatic BC particularly in cases with germline mutations in *BRCA1* or *BRCA2* genes, frequently associated with the TNBC subtype [64].

The yes-associated protein (YAP)/WW domain containing the transcription regulator 1 (TAZ)–TEA domain transcription factor (TEAD) complex is shown to be inversely correlated with *NORAD* expression in breast-invasive carcinoma in TCGA [65]. TEAD3 and TEAD4 are the anchor proteins of this complex, which are modulated by the Hippo signaling pathway, controlling cell growth and cancer progression [66]. TEAD4 was found to bind the *NORAD* promoter in the 5′ regulatory region of *NORAD* and silencing of *TEAD1/3/4* resulted in increased *NORAD* expression in the human TNBC Hs578T cell line [65]. YAP, TAZ and the NuRD-repressive complex [67] and other components, including metastasis-associated protein (*MTA1)* and chromodomain helicase DNA binding protein 4 (*CHD4*), were all recruited to that same region of *NORAD* promoter. Furthermore, silencing *MTA1* and *CHD4* led to further *NORAD* upregulation, confirming that YAP/TAZ and NuRD repress *NORAD* transcription. On the other hand, *NORAD* repression by the YAP/TAZ pathway contributes to the YAP/TAZ-mediated promotion of migration and invasion in the BC-mutated cell line Hs578 YAP 8SA [65], where YAP is inactive and cannot be phosphorylated [64][68]. *NORAD* silencing in the human ZR75 luminal BC cell line increased S100P association with the IQ motif containing GTPase activating protein 1 (IQGAP1) and TP53 proteins, while *NORAD* OE attenuated this interaction. In the human TNBC MDA-MB-231 cell line, the specific binding of S100P protein and *NORAD* was observed, with S100P OE reversing *NORAD* OE and S100P silencing counteracting *NORAD* depletion. A similar relationship was observed in vivo, where MDA-MB-231 *NORAD*-overexpressing cells, upon tail vein i.p. injection, formed fewer lung metastatic nodules compared to control or *NORAD*/S100P double KD cells. In this context, although *NORAD* is shown to be transcriptionally repressed by YAP/TAZ-TEAD, *NORAD* also sponges S100P to inhibit metastasis [65].

4. NORAD-Regulated Cytokines and Immune Cells

The tumor microenvironment (TME) plays a major role in BC progression and therapy response [69]. In particular, CD8 T immune cells are crucial in anticancer immune response [70], where a higher amount of CD8 T-infiltrating lymphocytes (TILs) predicts a better immunotherapy response [71] and high levels of CD8 T-cells in samples correlate with better BC prognosis [72]. *NORAD* expression in BC tissues is also proven to be correlated with the TME, immune infiltration and expression of immune checkpoint inhibitors [73]. The impact of *NORAD* in immune cell regulation during BC progression and in the therapy response will be highlighted below.

A study using data from TCGA, which divided BC samples into high and low CD8 T-cell numbers, revealed that *NORAD* expression was elevated in the low CD8 T-cell group and high-risk BC samples, with smaller OS rate. Moreover, *NORAD* was negatively correlated with the presence of CD8 T-cells, cytotoxic lymphocytes and T-cells in the tumor, while it was positively associated with the levels of fibroblasts, endothelial cells and neutrophils. *NORAD* expression was also negatively related to immune checkpoint genes such as lymphocyte-activating 3 (*LAG3*), T-cell immunoreceptor with Ig and ITIM domains (*TIGIT*), cytotoxic T-lymphocyte-associated protein 4 (*CTLA4*) and programmed cell death 1 (*PDCD1*) [72]. *NORAD* co-expresses with several targets of immune regulation signaling pathways such as cytokines and interleukins (ILs), as TGF-β, *IL-3*, *IL-4* and Type I Interferon [74]. These data show a connection between *NORAD* expression and immune cell regulation in BC, including CD8 T-cell numbers, which can potentially be modulated to improve therapy response.

In BC, *NORAD* expression was found to be preferentially related to macrophage regulation, which shows a preferential upregulation of M2-polarized protumoral CD206-expressing macrophages, in comparison with M1-polarized antitumoral CD68-expressing macrophages. A study revealed that macrophage polarization can be directed by TNBC cell line-derived exosome internalization. In comparison to macrophages incubated with exosomes derived from normal breast epithelium MCF-10A cells and *NORAD*-depleted MDA-MB-231 cells, MDA-MB-231-derived exosome co-culture with non-polarized macrophages resulted in higher levels of *NORAD* and expression of M2 markers (*CD163*; mannose receptor C type 2, *MRC2*; Arginase 1, *Arg1*) and lower expression of M1 markers (*CD80*; C-C motif chemokine ligand 2, *MCP-1*; nitric oxide synthase 2 *iNOS*). Moreover, macrophages previously incubated with *NORAD*-depleted MDA-MB-231-derived exosomes, when co-cultured with MDA-MB-231 cells, promoted several effects in the BC cells, including decreased expression of *NORAD*, reduced proliferation, migration and invasion and increased apoptosis. Moreover, silencing *NORAD* in macrophages decreased the expression of TGFB1 and phosphorylated Smad2 and 3, potentially through *miR-92b-3p*, that binds both *NORAD* and TGFB1. These results show that *NORAD* can contribute to the activation of macrophages that promote malignant behavior in BC cells [74].

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