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CRYβB2 enhances tumorigenesis through upregulation of nucleolin in triple negative breast cancer

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Abstract

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AUTHOR CONTRIBUTIONS

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COMPETING INTERESTS

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ADDITIONAL INFORMATION

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Expression of β-crystallin B2 (CRYβB2) is elevated in African American (AA) breast tumors. The underlying mechanisms of CRYβB2-induced malignancy and the association of CRYβB2 protein expression with survival have not yet been described. Here, we report that the expression of CRYβB2 in breast cancer cells increases stemness, growth, and metastasis. Transcriptomics data revealed that CRYβB2 upregulates genes that are functionally associated with unfolded protein response, oxidative phosphorylation, and DNA repair, while down-regulating genes related to apoptosis. CRYβB2 in tumors promotes de-differentiation, an increase in mesenchymal markers and cancer-associated fibroblasts, and enlargement of nucleoli. Proteome microarrays identified a direct interaction between CRYβB2 and the nucleolar protein, nucleolin. CRYβB2 induces nucleolin, leading to the activation of AKT and EGFR signaling. CRISPR studies revealed a dependency on nucleolin for the pro-tumorigenic effects of CRYβB2. Triple-negative breast cancer (TNBC) xenografts with upregulated CRYβB2 are distinctively sensitive to the nucleolin aptamer, AS-1411. Lastly, in AA patients, higher levels of nucleolar CRYβB2 in primary TNBC correlates with decreased survival. In summary, CRYβB2 is upregulated in breast tumors of AA patients and induces oncogenic alterations consistent with an aggressive cancer phenotype. CRYβB2 increases sensitivity to nucleolin inhibitors and may promote breast cancer disparity.

INTRODUCTION

Women of African American (AA) descent are more likely to die of breast cancer than are European American (EA) women [1–3]. The higher incidence of estrogen receptor-negative (ER^-) tumors with a less favorable prognosis among AA women contributes to the survival health disparity [4]. Also, studies have reported a worse long-term outcome for AA women with triple-negative breast cancer (TNBC) [2, 5].

Gene expression profiles have revealed differences in breast cancer between AA and EA women [6]. Expression of both phosphoserine phosphatase-like (*PSPHL*) and β-crystallin B2 (*CRYβB2*) was found to be higher in tumors from AA individuals, and this 2-gene signature correctly classified AA and EA breast [7], prostate [8], and colorectal [9] tumors. Recently, CRYβB2 and the related pseudogene, CRYβB2P1, were shown to have a function in TNBC [10].

CRYβB2 is among the major proteins of the vertebrate eye lens; mutations in this gene are associated with cataracts [11]. CRYβB2 improved the proliferation and survival of retinal ganglion cells [12], axons [13], and ovarian granulosa cells [14].

In this paper, we show that CRYβB2 induces aggressive behavior in breast cancer cells with low malignant potential. We investigated the role of a novel CRYβB2-interacting protein called nucleolin in CRYβB2-induced stemness, tumorigenesis, and metastasis. In patient breast tumors, CRYβB2 protein expression was associated with decreased survival, increased nucleolar size, and increased response to nucleolin inhibitors.

RESULTS

CRYβB2 is overexpressed in breast tumors of AA patients and is expressed in stem-like cells

Previously, it was reported that both *CRYβB2* and its pseudogene *CRYβB2P1* were overexpressed in AA breast tumors [15]. Analyzing breast tumor RNAseq data in The Cancer Genome Atlas (TCGA) database using their own custom scripts, Barrow et al., observed that *CRYβB2* tends to be differentially expressed in AA tumors [10]. Due to their partial sequence similarity and technical limitations in distinguishing between the two genes in expression arrays [10] and variability in data generated by different RNA-seq data analysis tools, there is a need to confirm whether both genes are indeed differentially expressed according to race. We repeated that analysis, using the BAM-slicing function available through the Genomic Data Commons Portal to download reads aligning to *CRYβB2* and/or *CRYβB2P1* (Fig. 1A). We observed that the pseudogene *CRYβB2P1* was even more highly expressed than *CRYβB2* and that the expression of both *CRYβB2* and *CRYβB2P1* was significantly upregulated in tumors of AA women ($n = 171$) compared to Asian ($n = 58$) and EA women ($n = 785$) (Fig. 1A, Supplementary Table S1 and Table S2). Moreover, tumors of the basal-like breast cancer subtype tended to express higher levels of *CRYβB2* and *CRYβB2P1* in comparison to the normal breast in all three race/ethnic groups (Supplementary Fig. S1A). While our findings are generally consistent with Barrow et al. [10] our data provide additional evidence that *CRYβB2P1* and *CRYβB2* are both up-regulated in AA tumors. To validate this observation, we performed Western blot analysis for CRYβB2 protein expression, and found that its levels were significantly higher ($P < 0.0001$) in primary estrogen receptor (ER)-negative tumors of AA patients ($n = 16$) when compared to EA patients ($n = 18$) (Fig. 1B).

Because CRYβB2 is involved in the regeneration of retinal cells [13], we asked whether the expression of CRYβB2 is related to stemness in normal breast epithelial cells. We found that *CRYβB2* was overexpressed exclusively in progenitor/stem cells ($CD44^+$) that were isolated from six normal breast tissues from AA women (Fig. 1C).

CRYβB2 promotes tumorigenesis of pre-invasive breast cancer cells

In order to identify the primary role of CRYβB2 in breast tumorigenesis, in the absence of the influence of additional oncogenes such as those driving proliferation in TNBC cells [10], we overexpressed this candidate oncogene in: (1) the immortalized human mammary epithelial cell line, MCF10A, (2) pre-invasive MCF10A-derived cell line, MCF10AT1 harboring a mutated HRAS oncogene [16], and (3) MCF10DCIS.COM (DCIS.COM) expressing both mutated HRAS and PIK3CA oncogenes [17] (Supplementary Fig. S1B). In all three cell lines overexpressing CRYβB2, we observed a nearly two-fold increase in the number of colonies formed compared to vector-transfected cells (Supplementary Fig. S1C). Thus, overexpression of CRYβB2 increased survival and cell proliferation of normal and pre-invasive breast cancer cells.

CRYβB2 was not sufficient to induce malignant transformation of MCF10A cells, as evidenced by the inability of the cells to form tumors in immunodeficient mice. On the

other hand, MCF10AT1 (Fig. 1D) and DCIS.COM (Fig. 1E) cells overexpressing CRYβB2 formed significantly larger tumors compared to control cells ($P < 0.0001$ and $P < 0.05$, respectively (Supplementary Fig. S1D), indicating an oncogenic function of CRYβB2 in the context of other genetic alterations. MCF10AT1-CRYβB2 cells metastasized to distal mammary glands, lungs, and bones (Fig. 1F, G, and Supplementary Fig. S1E). The lung lesions originating from CRYβB2$^+$ metastatic cells were significantly larger than those from CRYβB2$^-$ lesions (Fig. 1H), indicating accelerated growth attributable to CRYβB2 overexpression.

CRYβB2 increases nucleolar size, stromal recruitment, and epithelial to mesenchymal transition in breast tumors

CRYβB2 increased the growth of primary xenografts and induced the formation of lung metastasis. Therefore, we investigated the effect of CRYβB2 on the pathological and molecular features of these lesions. MCF10AT1-CRYβB2 tumors were less differentiated and resembled squamous cell carcinoma, while MCF10AT1-vector tumors were more differentiated and predominantly expressed features of adenocarcinoma (Fig. 2A). Fibrillarin staining revealed that MCF10AT1-CRYβB2 tumors harbor a larger number, and larger size nucleoli and nuclei compared to control tumors (Fig. 2A). CRYβB2 was expressed mainly in the nucleus, nucleolus, and cytoplasm of MCF10AT1-CRYβB2 tumor cells (Supplementary Fig. S2A). We also observed an increase in cancer-associated fibroblasts (CAF) which were CRYβB2$^-$ and alpha-smooth muscle actin (α-SMA)-positive in MCF10AT1-CRYβB2 tumors (Fig. 2A and Supplementary Fig. S2B). Expression of the mesenchymal marker, vimentin, increased in MCF10AT1-CRYβB2 tumors and was observed in both elongated mouse stromal cells and tumor cells with large nuclei (Fig. 2A and Supplementary Fig. S2C). Moreover, MCF10AT1-CRYβB2 tumors showed a decrease in expression of the epithelial marker, cytokeratin 18 (CK18, Supplementary Fig. S2D), and an increase in mesenchymal markers, Snail and Zeb-1 (Supplementary Fig. S2D). However, MCF10AT1-CRYβB2 tumors also expressed the basal epithelial cell markers CK14 (Fig. 2B) and integrin β4 (CD104, Supplementary Fig. S2D). Thus, the expression of CRYβB2 in low-malignant tumors appears to induce an EMT population that consists of hybrid epithelial- and mesenchymal-cell states [18]. In contrast, overexpression of CRYβB2 in the more invasive DCIS.COM cells generated tumors with an exclusively mesenchymal phenotype, with loss of the epithelial markers CK14, CK18, and E-cadherin (Fig. 2B and Supplementary Fig. S2E) and gain of mesenchymal markers, Snail and Zeb1 (Fig. 2B and Supplementary Fig. S2E). Loss of CK14 was described as the transition state from a hybrid- to more mesenchymal-tumor phenotype [18]. Together, these findings suggest that CRYβB2 induces features related to aggressive disease in pre-malignant and malignant human breast epithelial cells.

CRYβB2 increases cancer stem cell-like characteristics in breast tumors

Since we observed upregulated *CRYβB2* expression in the stem/progenitor cell population isolated from normal breast tissue from AA women (Fig. 1C), we sought to investigate its effect on the self-renewal of cancer cells. We observed that MCF10AT1 and DCIS. COM cells overexpressing CRYβB2 formed, on average, twice as many tumor-spheres compared to vector controls (Fig. 2C and Supplementary Fig. S2F). This suggested that CRYβB2 may

be involved in the expansion of cancer stem cells (CSC). Consistent with this hypothesis, MCF10AT1-CRYβB2 cells were significantly more efficient at engraftment into mammary fat pads of immunodeficient mice than vector control cells (Fig. 2D and Supplementary Fig. S2G).

To further strengthen these findings, we investigated whether CRYβB2 tumors have increased expression of CSC markers. In MCF10AT1-CRYβB2 tumors and in distal mammary gland metastasis we observed a significant decrease of CSC markers in differentiated cells ($EpCAM^+/CD24^+$) and an increase in stem/progenitor cells ($CD44^+/CD24^-$) (Fig. 2E and Supplementary Fig. S2H). Together, these results indicate that CRYβB2 expression in tumors resulted in an increased number of cells with CSC characteristics.

CRYβB2 regulates genes associated with enhanced malignant properties

To identify additional pathways of tumor aggression that are activated by CRYβB2, we performed large-scale gene expression profiling of MCF10AT1 and DCIS.COM cells overexpressing CRYβB2.

The analysis identified robust changes in gene expression in both MCF10AT1-CRYβB2 and DCIS.COM-CRYβB2 cells compared to control cells (Supplementary Fig. S2I). CRYβB2 decreased expression of genes with tumor suppressor function, such as *FMR1NB*, *ABCA5*, *Wnt7A*, *CLMN*, *NFKBIZ,* and *CDH4* and increased expression of oncogenic genes, such as *NPY1R* and *CAMP* in MCF10AT1 cells (Supplementary Fig. S2J, S2K).

A comprehensive search for the pathways that are affected by CRYβB2 identified unfolded protein response, oxidative phosphorylation, and DNA repair pathways as being enriched for CRYβB2-induced genes and the apoptosis pathway being enriched for genes down-regulated by CRYβB2 in the MCF10AT1 cells (Fig. 2F, Supplementary Fig. S2L, and Table S3). Accordingly, MCF10AT1-CRYβB2 tumors showed an increase in proteins induced by endoplasmic reticulum stress and proteins related to DNA damage/ repair and a decrease in apoptosis-related proteins (Supplementary Fig. S2M). The overexpression of CRYβB2 in DCIS.COM cells targeted different pathways, with activation of Wnt/β-catenin signaling, induction of mesenchymal phenotype, and downregulation of cell cycle control genes (Supplementary Table S4). These data are consistent with the induction of EMT and stemness in CRYβB2 expressing tumors (Fig. 2A) and the previously described correlation of CRYβB2 with the Wnt pathway signaling [19].

CRYβB2 interacts with proteins that regulate translation, cell proliferation, and invasion

In order to identify CRYβB2-interacting proteins and decipher additional mechanisms of CRYβB2-mediated induction of malignancy, we interrogated a human proteome microarray. The binding of either CRYβB2 or the bovine serum albumin (BSA) control to immobilized proteins was detected with a CRYβB2-specific antibody and visualized with a fluorescent-labeled secondary antibody (Fig. 3A). We identified several novel CRYβB2-interacting proteins which are known to be involved in the control of translation, such as poly(A) binding protein-interacting protein 1 (PAIP1), PAIP2, USO1, PUF60, ENDOU, nucleolin, ACBD3, cell death-PAK2, DNA damage-PPP4R3A, DNA repair-HNRNPD,

self-renewal-ACBD3 and proliferation-USO1, GRB2, ENDOU, and ANXA2 (Fig. 3B, Supplementary Fig. S3A and Table S5). We also observed that CRYβB2 interacted with several proteins involved in tumor cell invasion and metastasis (Supplementary Table S5). Using co-immunoprecipitation, we validated the binding of CRYβB2 to nucleolin, PAIP1, and GRB2 using lysates of MCF10AT1-CRYβB2 cells (Fig. 3C). The specificity of CRYβB2 binding to these proteins was supported by the fact that they were not immunoprecipitated using MCF10AT1-vector cell-lysate that lacked CRYβB2 expression (Fig. 3C). CRYβB2 also induced expression of the translational activator PAIP1 in MCF10AT1 cells (Fig. 3D). Therefore, we investigated if CRYβB2 can increase total protein synthesis using a puromycin-based pulse assay, SUnSET [20]. MCF10AT1 and DCIS.COM cells overexpressing CRYβB2 showed an increase in the incorporation of puromycin into nascent proteins, detected as an increase in the intensity of the smear in immunoblots using an anti-puromycin antibody (Fig. 3E). Moreover, the inhibitor of translation, homoharringtonine (HHT), was more effective in decreasing protein synthesis (Fig. 3E) and cell proliferation (average fold change 23 vs. 14 times, Fig. 3F) of MCF10AT1-CRYβB2 in comparison to control cells. Further, HHT treatment of MCF10AT1-CRYβB2 tumors resulted in significant inhibition of growth (Fig. 3G). These data showed that CRYβB2-regulation of protein synthesis is important for tumor growth.

CRYβB2 associates with the nucleus and endoplasmic reticulum

Next, we investigated the cellular localization of CRYβB2. We observed that CRYβB2 is detectable both in the cytoplasm and the nucleus (Fig. 3H). Our protein microarray analysis indicated that CRYβB2 is associated with proteins that regulate the translation and trafficking of proteins from the endoplasmic reticulum to the Golgi, like USO1 and ACBD3 (Fig. 3B). Hence, we determined if CRYβB2 localizes within these organelles. Confocal microscopy of labeled cells revealed that CRYβB2 associates with the PDI protein, an endoplasmic reticulum marker (Fig. 3H and Supplementary Fig. S3B). On the other hand, CRYβB2 did not associate with RCAS1, a Golgi marker (Supplementary Fig. S3C). These results suggest that in the cytoplasm, CRYβB2 associates with endoplasmic reticulum proteins, and trafficking of proteins from the endoplasmic reticulum to Golgi may have a role in CRYβB2-mediated promotion of malignancy.

CRYβB2 regulates the nucleolin pathway

The protein nucleolin which interacted with CRYβB2 (Fig. 3B, C) is mainly localized to the nucleolus and nucleus, where it regulates protein synthesis and cell proliferation [21]. CRYβB2 expression significantly increased the protein levels of nucleolin and activation of its associated proteins, including AKT and EGFR and the pro-survival Bcl2 protein in premalignant MCF10A-BRCA1-185delAG knock-in (KI) [22] (Fig. 4A and Supplementary Fig. S4A) and MCF10AT1 and DCIS.COM tumors (Fig. 4B and Supplementary Fig. S4B). With the exception of MCF10AT1 tumors, CRYβB2 expression resulted in decreased p53 levels (Fig. 4A, B, Supplementary Fig. S4A and S4C). In MCF10AT1 xenograft, overexpression of CRYβB2 resulted in activation of senescence proteins, such as p53, p21, and p16 (Fig. 4B, C and Supplementary Fig. S4C). Consequently, MCF10AT1- CRYβB2 tumors (3 out of 3) showed β-galactosidase staining, a marker of senescence (Fig. 4D and Supplementary Fig. S4D). No β-galactosidase staining was observed in tumors from

MCF10AT1- control cells (0 out of 3) (Fig. 4D and Supplementary Fig. S4D). These data suggest that in these low-malignant cells, in the presence of mutant HRAS, CRYβB2 increases p53 expression and induces senescence (Fig. 4C, 4D). Consistent with this finding, oncogenic RAS typically triggers cellular senescence [23].

Further analysis revealed that the knockout of nucleolin using CRISPR impaired AKT and EGFR activation in CRYβB2- overexpressing MCF10AT1 cells (Fig. 4E and Supplementary Fig. S4E). Knockdown of CRYβB2 in TNBC HCC1806 cells also resulted in a decrease of nucleolin, AKT, EGFR, and Bcl2 (Fig. 4E). In addition, CRYβB2 protein levels correlated with nucleolin expression in several TNBC (Fig. 4F) and ER^+ (Fig. 4G) cell lines. Together, the data provide strong support for a CRYβB2 function that increases nucleolin-related pathways in breast cancer cells.

Nucleolin mediates CRYβB2-oncogenic function

Nucleolin has been previously described to play a role in tumor cell proliferation [21], metastasis [24], and stem cell maintenance [25–27]. To address if nucleolin is involved in the CRYβB2-induction of malignancy, we used MCF10AT1-vector and -CRYβB2 cells knockout of nucleolin (Fig. 4E). Nucleolin loss significantly decreased colony formation (Fig. 5A and Supplementary Fig. S5A), sphere formation (Fig. 5B and Supplementary Fig. S5B) of MCF10AT1-CRYβB2 cells, and tumor size and weight (Fig. 5C and Supplementary Fig. S5C). On the other hand, nucleolin deficiency had a comparably smaller effect on sphere formation by MCF10AT1-vector cells (Fig. 5B and Supplementary Fig. S5B) and no effect on tumor growth of these cells (Fig. 5C and Supplementary Fig. S5C). Importantly, nucleolin-deficient MCF10AT1-CRYβB2 tumor-bearing mice showed significantly lower incidence and size of lung metastasis than MCF10AT1-CRYβB2-vector controls (Fig. 5D, Supplementary Fig. S5D, and S5E). These results provide evidence that nucleolin is, in large part, the mediator of CRYβB2-related induction of tumor cell proliferation, metastasis, and stem cell function. In line with these observations, the nucleolin aptamer AS-1411 inhibited the growth of CRYβB2 tumors but had no effect in tumors lacking CRYβB2 (Fig. 5E).

In order to address the role of CRYβB2 in TNBC, we knocked down this gene in MDA-MB-231 and HCC1806 cells (Fig. 4E). CRYβB2 knockdown decreased cell proliferation (Fig. 5F and Supplementary Fig. S5F) and tumor formation (Fig. 5G), a property similar to that observed in nucleolin-deficient cells (Supplementary Fig. S5G). Using a panel of TNBC cells, we observed that CRYβB2 protein expression inversely correlated with the sensitivity of the cells to the inhibitory nucleolin aptamer, AS-1411 (Fig. 5H). This data suggests that high CRYβB2 expression may predispose TNBC to the cytotoxic effects of nucleolin inhibitors. We next asked if CRYβB2 is functionally linked to the cellular response to AS-1411. We observed that the knockdown of CRYβB2 attenuated the inhibitory effect of AS-1411 on cell proliferation (Supplementary Fig. S5H), tumor growth (Fig. 5I), and metastasis (Fig. 5J). Consistent with CRYβB2 association with proteins that regulate invasion (Supplementary Table S5), the decrease of CRYβB2 in TNBC resulted in decreased metastasis (Fig. 5J). Collectively, these data suggested that CRYβB2 may be used as a predictor of TNBC prognosis and response to nucleolin inhibitors.

CRYβB2 expression is associated with poor TNBC outcome in AA women

We observed that CRYβB2 is overexpressed in ER^- tumors from AA patients (Fig. 1B), and promotes xenograft tumor growth (Fig. 1D). Therefore, we investigated whether CRYβB2 protein expression may correlate with survival of AA-TNBC patients (Supplementary Table S6).

In TNBC patients, CRYβB2 was expressed mainly in the nucleus, nucleolus, and cytoplasm and less often in the plasma membrane of tumor cells (Fig. 6A and Supplementary Fig. S6A). Furthermore, and consistent with our findings in tumor xenografts (Fig. 2A), nucleolar CRYβB2 expression correlated with an increase in nucleolar size (Fig. 6A, B). We observed nucleolar CRYβB2 expression in 81% (95% CI: 64–93%) of the tumors in AA TNBC patients who were never disease-free (NDF, $n = 32$) and in 77% (95% CI: 46–95%) of the metastasis ($n = 13$) (Fig. 6C). In contrast, most tumors from TNBC patients who remained disease-free (DF, n = 55) lacked nucleolar CRYβB2 expression ($n = 38$ or 69%; 95% CI: 55–81%) (Fig. 6C). We also observed nuclear CRYβB2 expression in 67% (95% CI: 48 – 82%) of the tumors in AA TNBC patients who were NDF ($n = 33$) but lack of nuclear CRYβB2 expression in most DF patients (34 out of 56 or 61%; 95% CI: 47–74%) (Fig. 6C).

Importantly, the presence of nucleolar and nuclear, but not cytoplasmic, CRYβB2 expression and nucleolar size in TNBC associated with a significant decrease in disease-free survival (DFS, $n = 86$) among AA women with TNBC (Fig. 6D, Supplementary Fig. S6B and Table S7). Nucleolar and nuclear CRYβB2 expression and nucleolar size in TNBC also tended to be associated with a decrease in overall survival (Supplementary Fig. S6B). In a multivariate model, nucleolar CRYβB2 expression is independently associated with poorer DFS (Supplementary Table S7). Collectively, these data show that CRYβB2 is associated with an increase in nucleolar size and poor prognosis in AA-TNBC patients.

DISCUSSION

CRYβB2 is expressed in both tumors and normal breast tissue of AA women at higher levels in comparison to those in EA women [6]. However, the role of CRYβB2 in tumor initiation and progression remains poorly understood. In this paper, we showed that CRYβB2 is overexpressed in tumor-initiating cells of the normal breast in AA women, suggesting a role of CRYβB2 in the early events of tumor formation, and in recurrence following treatment. In line with this, CRYβB2 activated the nucleolin-AKT axis, resulting in the inhibition of p53 in premalignant breast cancer cells with BRCA1-185delAG mutation. Mutations in the BRCA1 gene are common in breast cancer in AA patients with early-onset disease, family history of breast cancer, or TNBC [28, 29].

CRYβB2 induced the growth of tumors with a single hit mutation in the MAPK pathway. Characterization of the role of CRYβB2 in pre-invasive breast cancer cells revealed several features associated with an increase in malignancy that correlated with a worse outcome in patients [30]. Tumors arising from breast cancer cells with CRYβB2 overexpression were less differentiated, with an increased size of nuclei and nucleoli, an increased number of tumor-associated fibroblasts, EMT markers, progenitor/stem cell content, and metastasis.

Consistent with these features, CRYβB2 interacted with several proteins that regulate cell proliferation and invasion. Similarly, CRYβB2 was recently shown to increase the expression of genes associated with EMT in a TNBC xenograft model [10]. TNBC tumors with high CRYβB2 expression showed increased levels of interleukin-6 [10], which could be a result of increased recruitment of CAF [31].

CRYβB2 mutations lead to apoptosis in human lens epithelial cells due to activation of the unfolded protein response (UPR) in the lumen of the endoplasmic reticulum [32]. Gene expression array and pathway analysis revealed that CRYβB2 activated UPR and DNA repair pathways and decreased apoptosis pathways, possibly as a way to control the function of unfolded proteins in the endoplasmic reticulum during rapid proliferation of cells. In line with these observations, CRYβB2 is localized to the endoplasmic reticulum and is associated with proteins that regulate the translation and trafficking of proteins from the endoplasmic reticulum to the Golgi. CRYβB2 cells induced the stimulator of translation PAIP1 [33] and total protein synthesis. CRYβB2-overexpressing tumors were sensitive to the protein inhibitor HHT. HHT is a drug approved for the treatment of chronic myeloid leukemia [34] and may target TNBC, as shown in preclinical studies [35].

In addition to the regulation of ribosome production, the nucleolus regulates genome stability, cell-cycle control, cellular senescence, and stress responses, driving cancer growth and proliferation [36]. Supporting its function as a regulator of protein synthesis, we observed that CRYβB2 binds to nucleolin and regulates its expression and function in both TNBC and ER^+ cancer cells. Nucleolin is a multifunctional protein that is mainly localized in the nucleolus, where it regulates ribosome biogenesis and contributes to cell proliferation [21]. Altered nucleolin expression and localization results in oncogenic effects, such as stabilization of *AKT*, *Bcl-2*, *Bcl-XL*, and *IL-2* mRNAs [37–39]. Phospho-AKT was reduced by nucleolin knockdown in prostate cancer cells [40]. The overexpression of EGFR and nucleolin lead to receptor dimerization, phosphorylation, and anchorage-independent growth [41]. The p85α was shown to promote nucleolin transcription and subsequently enhance EGFR mRNA stability and EGF-induced malignant cellular transformation [42]. Nucleolin is also involved in the post-transcriptional inhibition of p53 [43]. We demonstrated that nucleolin is the mediator of the effects of CRYβB2 on increases in AKT, EGFR, and Bcl2 activation and decrease of p53 protein. In low-malignant cells with a single mutation in the RAS pathway, CRYβB2 activation resulted in increases of p53, p21, and p16, leading to senescence. Oncogenic RAS typically triggers cellular senescence, a state of irreversible cell growth arrest [23]. However, senescence can also promote cancer development by altering the cellular microenvironment through a senescence-associated secretory phenotype [44].

Nucleolin maintains embryonic [25] and breast cancer [27] stem cells function. Nucleolin was also implicated in EMT [45] and migration/ invasion of tumor cells [24]. Accordingly, we observed that nucleolin mediates CRYβB2-induced increase of tumor growth, stemness, and metastasis. We showed that CRYβB2 determined TNBC sensitivity to anti-nucleolin therapies, including the nucleolin aptamer AS-1411. Results from clinical trials show that AS-1411 has a safety profile and clinical activity [46], identifying it as a potential therapeutic that is worthy of further investigation in AA TNBC.

According to CRYβB2-regulation of nucleolin, we observed that nucleolar CRYβB2 expression correlated with an increase in nucleolar size in TNBC patients. Nucleolar size has been used as a predictive and prognostic biomarker in chemotherapy regimens [47] and clinical outcomes [48]. Nucleolar, and to a lesser extent, nuclear CRYβB2 expression most effectively identifies AA-TNBC patients who are less likely to survive. In fact, we also observed a high frequency of nucleolar CRYβB2 expression in metastatic lesions. In addition, we showed that CRYβB2 expression also correlated with nucleolin expression in ER^+ tumor cells. The role of CRYβB2 to predict the survival of patients with ER^+ tumors remains to be determined.

In summary, CRYβB2 induces EMT, stemness, and protein synthesis in TNBC through the regulation of the nucleolin-AKT axis, contributing to an increase in tumor growth and metastasis. CRYβB2 is overexpressed in tumors of AA patients and could be explored further as a biomarker of disease outcome and to assess response to nucleolin and translation inhibitors.

MATERIALS AND METHODS

Patient samples, cell lines, and reagents

The breast tissue of women undergoing reduction mammoplasty and primary tumors from women undergoing treatment were provided by the Johns Hopkins Surgical Pathology Department, following patient consent under protocols approved by the institutional review board. $CD24^+$ and $CD44^+$ cells were isolated from fresh normal breast tissue using magnetic beads as described previously [49]. RNA and protein were extracted from normal and breast cancer tissue [50]. Tissue microarrays (TMAs) were provided by Dr. Naab and Dr. Kanaan from Howard University. Briefly, the TMAs were constructed by Pantomics (Fairfield, CA) using FFPE tumor blocks from primary TNBC (87) and axillary lymph nodes (15) from AA women (Supplementary Table S6). Breast cancer cells were obtained from the American Type Culture Collection. MCF10A-BRCA1-185delAG knock-in cells were obtained from Dr. Ben H. Park. MCF10AT1 and DCIS.COM cells were obtained from the Barbara Ann Karmanos Cancer Institute. Cells were authenticated using short tandem repeat (STR) profiling and tested for mycoplasma using MycoAlert PLUS mycoplasma detection kit (Lonza). Homoharringtonine (HHT) was purchased from Sigma Aldrich. The nucleolin aptamer AS-1411 (5′-GGTGGTGGTGGTTGTGGTGGTGGTGG) and CRO control (5′-CCTCCTCCTCCTTCTCCTCCTCCTCC) were purchased from Integrated DNA technologies.

Constructs

CRYβB2 coding sequence was cloned into a lentivirus vector (Addgene) using the Gateway Technology System (Thermo Fisher). MCF10A, MCF10AT1, and DCIS.COM cells overexpressing luciferase and CRYβB2 were generated following lentivirus infection. For immunofluorescence, MCF10AT1 cells were infected with lentivirus containing the CRYβB2 sequence tagged with the myc-DDK (flag) sequence (Origene). For CRISPR knockout, nucleolin and CRYβB2 guide RNAs were designed using sgRNA online web page from Broad Institute and cloned into Lenticrispr V2 (Addgene). 293T cells were

transfected with the lentivirus constructs using Lipofectamine and viruses were used to infect cancer cells [50].

Xenograft and limiting dilution assay

All animal studies were performed according to the guidelines and approval of the Animal Care Committee of the Johns Hopkins School of Medicine. Xenografts of DCIS.COM and MCF10AT1 cells expressing vector control and CRYβB2 and nucleolin knockout constructs and HCC1806 control and CRYβB2 knockdown were established in 6 to 8-week old NOD-scid IL2Rgnull (NSG) female mice (from an in-house colony at Hopkins) by injecting 5×10^6 and 2×10^6 tumor cells, respectively, into the fourth mammary gland. When the average tumor volume reached 100 mm^3, mice were randomized into various groups, and treatment was blinded. Mice were treated for 5 weeks with HHT (1 mg/kg) or saline as a vehicle for 5 days/ week i.p. Mice were injected daily, by the intraperitoneal (i.p.) route for the indicated times with 100 μL of a sterile PBS solution containing 22 μg of AS-1411 or CRO. For limiting dilution assays, MCF10AT1 cells were injected at limiting dilutions (5×10^6 – 1×10^5) into mammary fat pads [50]. The CSC frequency was estimated using Extreme Limiting Dilution Analysis (ELDA). Bioluminescence imaging was performed using an IVIS system [51].

Western blot, immunohistochemistry, immunofluorescence, and senescence

Western blot, immunohistochemistry (IHC) [50], and immunofluorescence (IF) [52] were performed as previously described using antibodies against CRYβB2, EMT markers, apoptosis, and DNA damage (Supplementary Methods). For IF, the slides were probed with the following primary antibodies: CRYβB2 (Thermo Fisher), CRYBB2-myc DDK flag (Cell Signaling Technology), organelle-specific antibodies, and nuclear staining (Hoechst; Fisher Scientific) [52]. ImageJ was used for quantification. Senescence assay was performed by beta-galactosidase staining of tumors using a commercial kit (Cell Signaling).

Bioinformatics analysis

BAM files corresponding to RNAseq results for TCGA breast tumors were remotely sliced to include reads for CRYβB2 and CRYβB2P1 and downloaded from the Genomic Data Commons data portal. Data from total read counts for each sample were obtained by summing over all genes in the HT-seq count files.

Transcriptome array

Human GE 4 × 44 K microarray (G4845A, Agilent) was performed by the Microarray Core at Johns Hopkins using RNA from MCF10AT1 and DCIS.COM- CRYβB2 and control cells ($n = 3$). After pre-processing using GenomeStudio, the data was analyzed with R. Pathway Analysis was performed using gene set variant analysis on Hallmark gene sets defined by Molecular Signatures Database.

Human proteome microarray

HuProt™ Human Proteome Microarray chips (CDI Labs) were hybridized with MCF10AT1-CRYβB2 cell lysate or BSA control. CRYβB2 (Santa Cruz Biotechnology) and Cy5-labeled secondary antibodies were used to detect proteins that interact with CRYβB2 protein.

Statistical analysis

Two-tailed nonparametric Mann–Whitney Test and parametric Student's *T*-test were performed on pairwise combinations of non-normally and normally distributed data, respectively, to determine statistical significance defined as $P < 0.05$. Log-rank test was performed using GraphPad Prism. Multivariate Cox proportional hazards analysis was calculated using the R program.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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REFERENCES

1. Menashe I, Anderson WF, Jatoi I, Rosenberg PS. Underlying causes of the black-white racial disparity in breast cancer mortality: a population-based analysis. J Natl Cancer Inst 2009;101:993–1000. [PubMed: 19584327]
2. Dietze EC, Sistrunk C, Miranda-Carboni G, O'Regan R, Seewaldt VL. Triple-negative breast cancer in African-American women: disparities versus biology. Nat Rev Cancer 2015;15:248–54. [PubMed: 25673085]
3. DeSantis CE, Fedewa SA, Goding Sauer A, Kramer JL, Smith RA, Jemal A. Breast cancer statistics, 2015: convergence of incidence rates between black and white women. CA: Cancer J Clin 2016;66:31–42. [PubMed: 26513636]
4. Bauer KR, Brown M, Cress RD, Parise CA, Caggiano V. Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and HER2-negative invasive breast cancer, the so-called triple-negative phenotype: a population-based study from the California cancer Registry. Cancer 2007;109:1721–8. [PubMed: 17387718]
5. Cho B, Han Y, Lian M, Colditz GA, Weber JD, Ma C, et al. Evaluation of racial/ethnic differences in treatment and mortality among women with triple-negative breast cancer. JAMA Oncology 2021;7:1016–23. [PubMed: 33983438]
6. Field LA, Love B, Deyarmin B, Hooke JA, Shriver CD, Ellsworth RE. Identification of differentially expressed genes in breast tumors from African American compared with Caucasian women. Cancer 2012;118:1334–44. [PubMed: 21800289]
7. Martin DN, Boersma BJ, Yi M, Reimers M, Howe TM, Yfantis HG, et al. Differences in the tumor microenvironment between African-American and European-American breast cancer patients. PLoS One 2009;4:e4531. [PubMed: 19225562]
8. Wallace TA, Prueitt RL, Yi M, Howe TM, Gillespie JW, Yfantis HG, et al. Tumor immunobiological differences in prostate cancer between African-American and European-American men. Cancer Res 2008;68:927–36. [PubMed: 18245496]
9. Jovov B, Araujo-Perez F, Sigel CS, Stratford JK, McCoy AN, Yeh JJ, et al. Differential gene expression between African American and European American colorectal cancer patients. PLoS One 2012;7:e30168. [PubMed: 22276153]

10. Barrow MA, Martin ME, Coffey A, Andrews PL, Jones GS, Reaves DK, et al. A functional role for the cancer disparity-linked genes, CRYbetaB2 and CRY-betaB2P1, in the promotion of breast cancer. Breast cancer Res: BCR 2019;21:105. [PubMed: 31511085]
11. Graw J Genetics of crystallins: cataract and beyond. Exp Eye Res 2009;88:173–89. [PubMed: 19007775]
12. Anders F, Teister J, Liu A, Funke S, Grus FH, Thanos S, et al. Intravitreal injection of beta-crystallin B2 improves retinal ganglion cell survival in an experimental animal model of glaucoma. PLoS One 2017;12:e0175451. [PubMed: 28384305]
13. Liedtke T, Schwamborn JC, Schroer U, Thanos S. Elongation of axons during regeneration involves retinal crystallin beta b2 (crybb2). Mol Cell Proteom: MCP 2007;6:895–907. [PubMed: 17264069]
14. Gao Q, Sun LL, Xiang FF, Gao L, Jia Y, Zhang JR, et al. Crybb2 deficiency impairs fertility in female mice. Biochemical Biophysical Res Commun 2014;453:37–42.
15. Sturtz LA, Melley J, Mamula K, Shriver CD, Ellsworth RE. Outcome disparities in African American women with triple negative breast cancer: a comparison of epidemiological and molecular factors between African American and Caucasian women with triple negative breast cancer. BMC cancer 2014;14:62. [PubMed: 24495414]
16. Dawson PJ, Wolman SR, Tait L, Heppner GH, Miller FR. MCF10AT: a model for the evolution of cancer from proliferative breast disease. Am J Pathol 1996;148:313–9. [PubMed: 8546221]
17. Miller FR, Santner SJ, Tait L, Dawson PJ. MCF10DCIS.com xenograft model of human comedo ductal carcinoma in situ. J Natl Cancer Inst 2000;92:1185–6.
18. Pastushenko I, Brisebarre A, Sifrim A, Fioramonti M, Revenco T, Boumahdi S, et al. Identification of the tumour transition states occurring during EMT. Nature 2018;556:463–8. [PubMed: 29670281]
19. Paulucci DJ, Sfakianos JP, Skanderup AJ, Kan K, Tsao CK, Galsky MD, et al. Genomic differences between black and white patients implicate a distinct immune response to papillary renal cell carcinoma. Oncotarget 2017;8:5196–205. [PubMed: 28029648]
20. Schmidt EK, Clavarino G, Ceppi M, Pierre P. SUnSET, a nonradioactive method to monitor protein synthesis. Nat Methods 2009;6:275–7. [PubMed: 19305406]
21. Bugler B, Caizergues-Ferrer M, Bouche G, Bourbon H, Amalric F. Detection and localization of a class of proteins immunologically related to a 100-kDa nucleolar protein. Eur J Biochem 1982;128:475–80. [PubMed: 7151790]
22. Konishi H, Mohseni M, Tamaki A, Garay JP, Croessmann S, Karnan S, et al. Mutation of a single allele of the cancer susceptibility gene BRCA1 leads to genomic instability in human breast epithelial cells. Proc Natl Acad Sci USA 2011;108:17773–8. [PubMed: 21987798]
23. Tu Z, Aird KM, Zhang R. RAS, cellular senescence and transformation: the BRCA1 DNA repair pathway at the crossroads. Small GTPases 2012;3:163–7. [PubMed: 22751483]
24. Chen Z, Xu X. Roles of nucleolin. Focus on cancer and anti-cancer therapy. Saudi Med J 2016;37:1312–8. [PubMed: 27874146]
25. Yang A, Shi G, Zhou C, Lu R, Li H, Sun L, et al. Nucleolin maintains embryonic stem cell self-renewal by suppression of p53 protein-dependent pathway. J Biol Chem 2011;286:43370–82. [PubMed: 22013067]
26. Mahotka C, Bhatia S, Kollet J, Grinstein E. Nucleolin promotes execution of the hematopoietic stem cell gene expression program. Leukemia 2018;32:1865–8. [PubMed: 29572507]
27. Fonseca NA, Rodrigues AS, Rodrigues-Santos P, Alves V, Gregorio AC, Valerio-Fernandes A, et al. Nucleolin overexpression in breast cancer cell sub-populations with different stem-like phenotype enables targeted intracellular delivery of synergistic drug combination. Biomaterials 2015;69:76–88. [PubMed: 26283155]
28. Churpek JE, Walsh T, Zheng Y, Moton Z, Thornton AM, Lee MK, et al. Inherited predisposition to breast cancer among African American women. Breast cancer Res Treat 2015;149:31–39. [PubMed: 25428789]
29. Atchley DP, Albarracin CT, Lopez A, Valero V, Amos CI, Gonzalez-Angulo AM, et al. Clinical and pathologic characteristics of patients with BRCA-positive and BRCA-negative breast cancer. J Clin Oncol: Off J Am Soc Clin Oncol 2008;26:4282–8.

30. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell 2000;100:57–70. [PubMed: 10647931]
31. Gieniec KA, Butler LM, Worthley DL, Woods SL. Cancer-associated fibroblasts-heroes or villains? Br J Cancer 2019;121:293–302. [PubMed: 31289350]
32. Li L, Fan DB, Zhao YT, Li Y, Kong DQ, Cai FF, et al. Two novel mutations identified in ADCC families impair crystallin protein distribution and induce apoptosis in human lens epithelial cells. Sci Rep 2017;7:17848. [PubMed: 29259299]
33. Lv Y, Zhang K, Gao H. Paip1, an effective stimulator of translation initiation, is targeted by WWP2 for ubiquitination and degradation. Mol Cell Biol 2014;34:4513–22. [PubMed: 25266661]
34. Alvandi F, Kwitkowski VE, Ko CW, Rothmann MD, Ricci S, Saber H, et al. U.S. food and drug administration approval summary: omacetaxine mepesuccinate as treatment for chronic myeloid leukemia. Oncologist 2014;19:94–99. [PubMed: 24309980]
35. Yakhni M, Briat A, El Guerrab A, Furtado L, Kwiatkowski F, Miot-Noirault E, et al. Homoharringtonine, an approved anti-leukemia drug, suppresses triple negative breast cancer growth through a rapid reduction of anti-apoptotic protein abundance. Am J Cancer Res 2019;9:1043–60. [PubMed: 31218111]
36. Carotenuto P, Pecoraro A, Palma G, Russo G, Russo. A therapeutic approaches targeting nucleolus in cancer. Cells 2019;8:1090. [PubMed: 31527430]
37. Otake Y, Soundararajan S, Sengupta TK, Kio EA, Smith JC, Pineda-Roman M, et al. Overexpression of nucleolin in chronic lymphocytic leukemia cells induces stabilization of bcl2 mRNA. Blood 2007;109:3069–75. [PubMed: 17179226]
38. Chen CY, Gherzi R, Andersen JS, Gaietta G, Jurchott K, Royer HD, et al. Nucleolin and YB-1 are required for JNK-mediated interleukin-2 mRNA stabilization during T-cell activation. Genes Dev 2000;14:1236–48. [PubMed: 10817758]
39. Abdelmohsen K, Tominaga K, Lee EK, Srikantan S, Kang MJ, Kim MM, et al. Enhanced translation by Nucleolin via G-rich elements in coding and non-coding regions of target mRNAs. Nucleic acids Res 2011;39:8513–30. [PubMed: 21737422]
40. Shin SH, Lee GY, Lee M, Kang J, Shin HW, Chun YS, et al. Aberrant expression of CITED2 promotes prostate cancer metastasis by activating the nucleolin-AKT pathway. Nat Commun 2018;9:4113. [PubMed: 30291252]
41. Di Segni A, Farin K, Pinkas-Kramarski R. Identification of nucleolin as new ErbB receptors-interacting protein. PLoS One 2008;3:e2310. [PubMed: 18523588]
42. Xie Q, Guo X, Gu J, Zhang L, Jin H, Huang H, et al. p85alpha promotes nucleolin transcription and subsequently enhances EGFR mRNA stability and EGF-induced malignant cellular transformation. Oncotarget 2016;7:16636–49. [PubMed: 26918608]
43. Takagi M, Absalon MJ, McLure KG, Kastan MB. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. Cell 2005;123:49–63. [PubMed: 16213212]
44. Zeng S, Shen WH, Liu L. Senescence and Cancer. Cancer Transl Med 2018;4:70–74. [PubMed: 30766922]
45. Yang Y, Yang C, Zhang J. C23 protein meditates bone morphogenetic protein-2-mediated EMT via up-regulation of Erk1/2 and Akt in gastric cancer. Med Oncol 2015;32:76. [PubMed: 25698539]
46. Rosenberg JE, Bambury RM, Van Allen EM, Drabkin HA, Lara PN Jr., Harzstark AL, et al. A phase II trial of AS1411 (a novel nucleolin-targeted DNA aptamer) in metastatic renal cell carcinoma. Investigational N. drugs 2014;32:178–87.
47. Derenzini M, Trere D, Pession A, Montanaro L, Sirri V, Ochs RL. Nucleolar function and size in cancer cells. Am J Pathol 1998;152:1291–7. [PubMed: 9588897]
48. Derenzini M, Nardi F, Farabegoli F, Ottinetti A, Roncaroli F, Bussolati G. Distribution of silver-stained interphase nucleolar organizer regions as a parameter to distinguish neoplastic from nonneoplastic reactive cells in human effusions. Acta cytologica 1989;33:491–8. [PubMed: 2473585]
49. Shipitsin M, Campbell LL, Argani P, Weremowicz S, Bloushtain-Qimron N, Yao J, et al. Molecular definition of breast tumor heterogeneity. Cancer Cell 2007;11:259–73. [PubMed: 17349583]
50. Merino VF, Nguyen N, Jin K, Sadik H, Cho S, Korangath P, et al. Combined treatment with epigenetic, differentiating, and chemotherapeutic agents cooperatively targets tumor-initiating cells in triple-negative breast cancer. Cancer Res 2016;76:2013–24. [PubMed: 26787836]

51. Pomper MG, Hammond H, Yu X, Ye Z, Foss CA, Lin DD, et al. Serial imaging of human embryonic stem-cell engraftment and teratoma formation in live mouse models. Cell Res 2009;19:370–9. [PubMed: 19114988]

52. Foss CA, Kulik L, Ordonez AA, Jain SK, Michael Holers V, Thurman JM, et al. SPECT/CT Imaging of Mycobacterium tuberculosis Infection with [(125)I]anti-C3d mAb. Mol Imaging Biol 2019;21:473–81. [PubMed: 29998399]

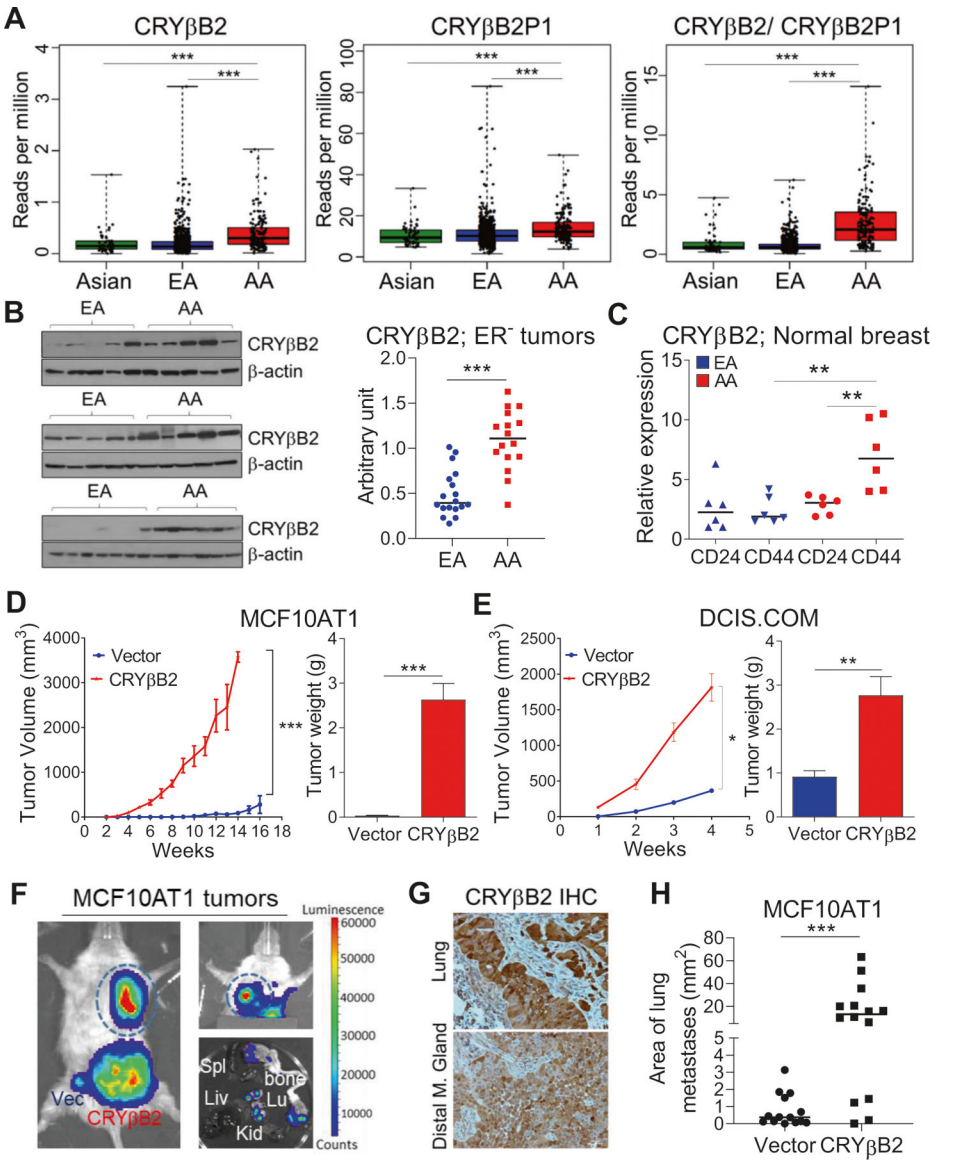

Fig. 1. CRYβB2 is overexpressed in AA tumors and promotes tumorigenesis.

A Boxplot representation of *CRYβB2* and *CRYβB2P1* mRNA expression by analysis of RNA seq data from TCGA in breast tumors from Asian (n = 58), European American (EA) (n = 785), and African American (AA) (n = 171) and normal breast tissue from Asian (n = 4), European American (n = 84) and African American (n = 17). Wilcoxon rank-sum test was used to calculate differences in expression. **B** Western blot determination in triplicate and ImageJ quantification of CRYβB2 protein expression in estrogen receptor (ER) negative tumors from AA (n = 16) and EA women (n = 18). β-actin: loading control. **C** *CRYβB2* mRNA expression in CD24 (differentiated) and CD44 (stem cell) populations isolated from AA and EA normal breast (n = 6 each). Tumor volume and weight of MCF10AT1 (**D**) and DCIS.COM (**E**) - CRYβB2 and control xenografts (n = 5 each). Standard error of mean (SEM) is reported. **F** Bioluminescence imaging of MCF10AT1-CRYβB2 and control tumors and distant metastases to the mammary gland, lung, and bone. **G** CRYβB2 IHC of lung and

mammary gland. **H** Image J quantification of lung area with metastases. Mann-Whitney test was performed. *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$.

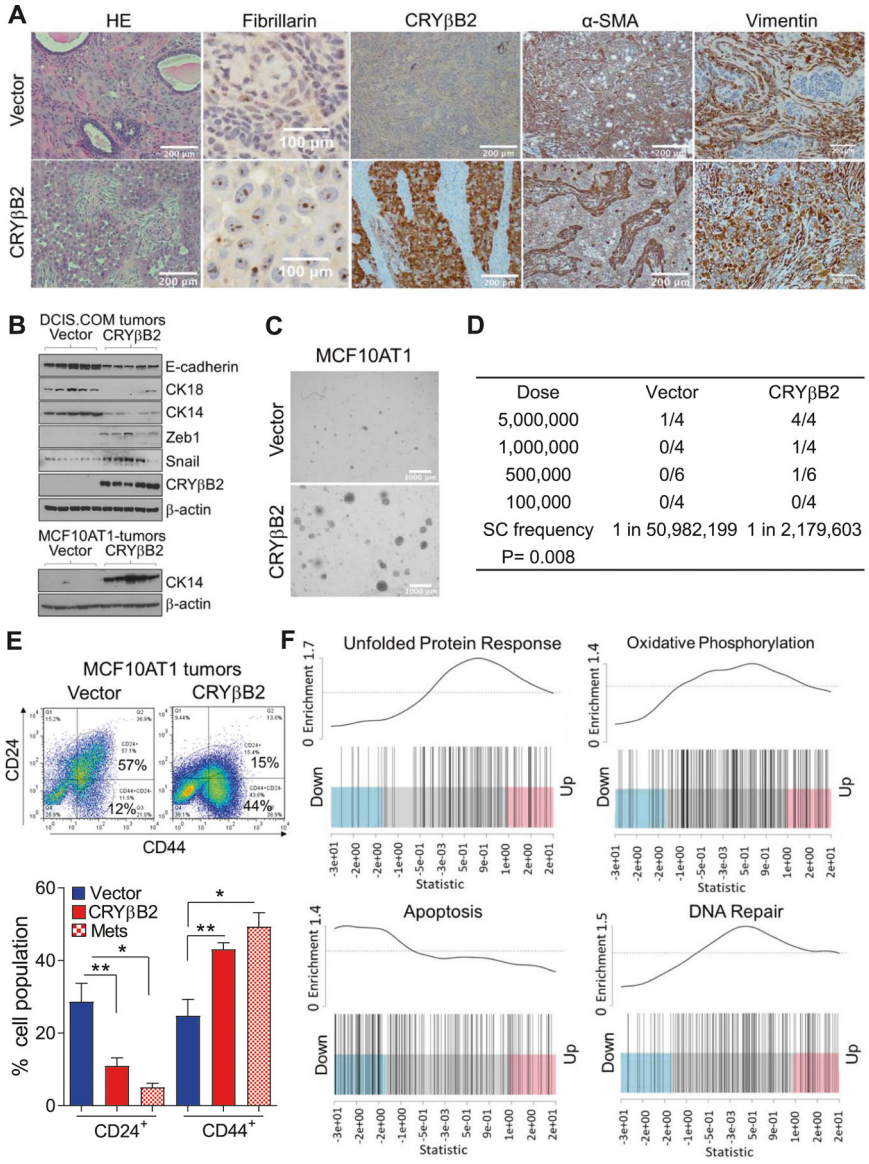

Fig. 2. CRYβB2 tumors present features of aggressive breast cancer.

A Morphological analysis of MCF10AT1-CRYβB2 xenografts following 16 weeks of growth. FFPE sections of tumors were stained with hematoxylin and eosin (HE); IHC for fibrillarin, CRYβB2, mouse alpha-smooth muscle actin (α-SMA), and vimentin. **B** Western blot determination in triplicate of epithelial and mesenchymal markers in DCIS.COM and MCF10AT1 tumors (n = 5 each). β-actin: loading control. **C** Mammosphere assay in triplicate using MCF10AT1-CRYβB2 and control cells. **D** ELDA software was used to calculate the cancer stem cell (CSC) frequency in the limiting-dilution assay of MCF10AT1-CRYβB2 and control cells at week 3 of tumor growth. Different cell doses and tumor incidence at each dose is shown. **E** Flow cytometry determination of CD44+/CD24− (CSC) and CD44+/CD24+/EpCAM+ (differentiated) populations in MCF10AT1-CRYβB2 and control tumors (n=10) and metastases within the distal mammary gland (n = 3). The student's t-test was performed, and results are expressed as mean ± SEM. **F** Gene Set

Variation Analysis (GSVA) scores of gene set analysis (GSEA) hallmark gene sets for MCF10AT1-CRYβB2 cells in comparison to vector ($n = 3$ each). Representative pathways are shown. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

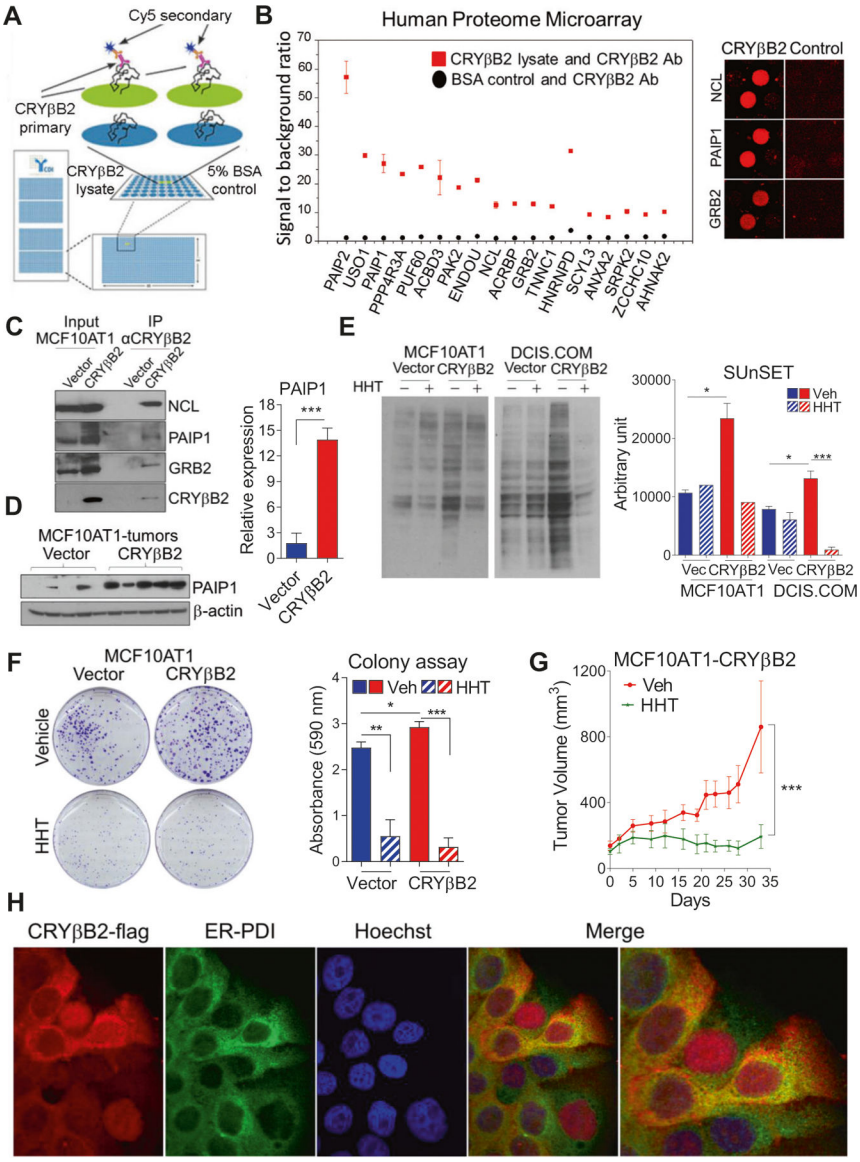

Fig. 3. CRYβB2 interactome and regulation of translation.

A Scheme of HuProt™ human proteome microarray-based discovery. **B** Identification of CRYβB2-associated proteins and representative images of binding of CRYβB2 antibody to nucleolin (NCL), PAIP1, and GRB2 in the presence of MCF10AT1-CRYβB2 lysate or 5% BSA control. **C** Co-immunoprecipitation of CRYβB2 and its associated proteins in duplicate, using total protein lysate (input) or immunoprecipitated complexes (IP) from MCF10AT1-vector and -CRYβB2 cells. **D** Western blot determination in triplicate of PAIP1 protein in MCF10AT1-vector and -CRYβB2 tumors. **E** SUnSET measurement of protein synthesis in triplicate in MCF10AT1 and DCIS.COM- CRYβB2 and control cells treated with homoharringtonine (HHT, 50 nM) for 48h, followed by puromycin (1 μM) for 30 min. Protein synthesis was detected by immunoblotting with an anti-puromycin antibody. **F** Proliferation assay in quadruplicate and quantification of crystal violet staining (absorbance at 590 nm) of MCF10AT1-vector and -CRYβB2 cells treated with vehicle and

or homoharringtonine (HHT, 10 nM) for 10 days. **G** Mean tumor volume±SEM of 5 mice per group bearing MCF10AT1-CRYβB2 xenografts and treated for 5 weeks with vehicle (saline) or HHT (1 mg/kg, i.p). **H** Immunofluorescence staining of MCF10AT1-CRYβB2 cells with anti-CRYβB2-flag (red), anti-protein disulfide isomerase (PDI) (green), and Hoechst (blue). The merge of the fluorescent channels is shown (right). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

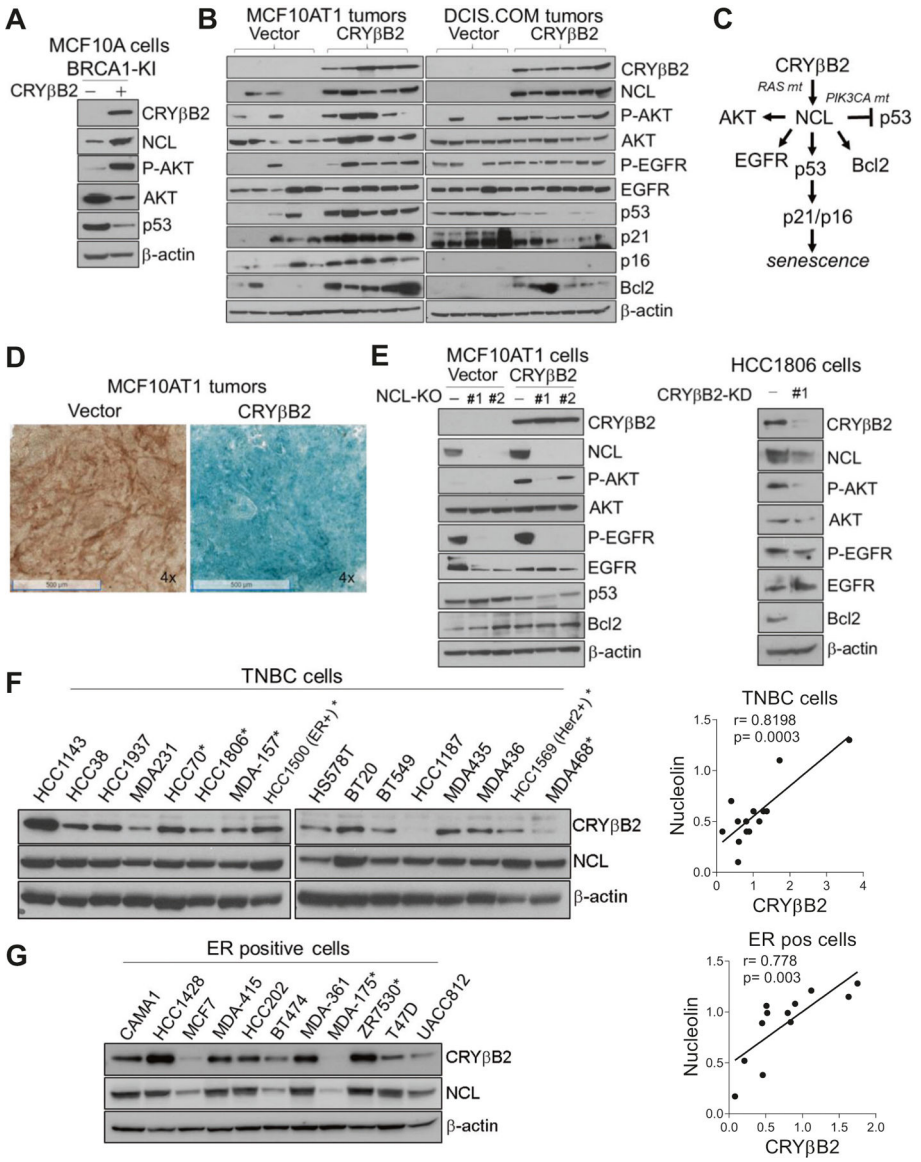

Fig. 4. CRYβB2 associates to nucleolin and regulate its pathway.

A Western blot determination of CRYβB2, nucleolin (NCL), and downstream effectors protein levels in (**A**) MCF10A-BRCA1-185delAG knock-in (KI) [22], and (**B**) MCF10AT1 and DCIS.COM tumors overexpressing CRYβB2 and control plasmids (n = 5–6 tumors each). **C** Scheme of Western blot conclusions of CRYβB2- nucleolin pathway. **D** Senescence beta-galactosidase staining in duplicate of MCF10AT1-vector and -CRYβB2 tumors (n = 3). Western blot determination of nucleolin, CRYβB2, and downstream effectors protein expression in MCF10AT1-vector and -CRYβB2 cells control and NCL knockout (KO) and HCC1806 cells control and CRYβB2 knockdown (KD) (**E**); TNBC (**F**) and estrogen receptor $(ER)^+$ (**G**) cells. (*) Cell lines from AA patients. Direct correlation of protein expression, Pearson correlation coefficient (r), and the p-value are shown. β-actin: loading control. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

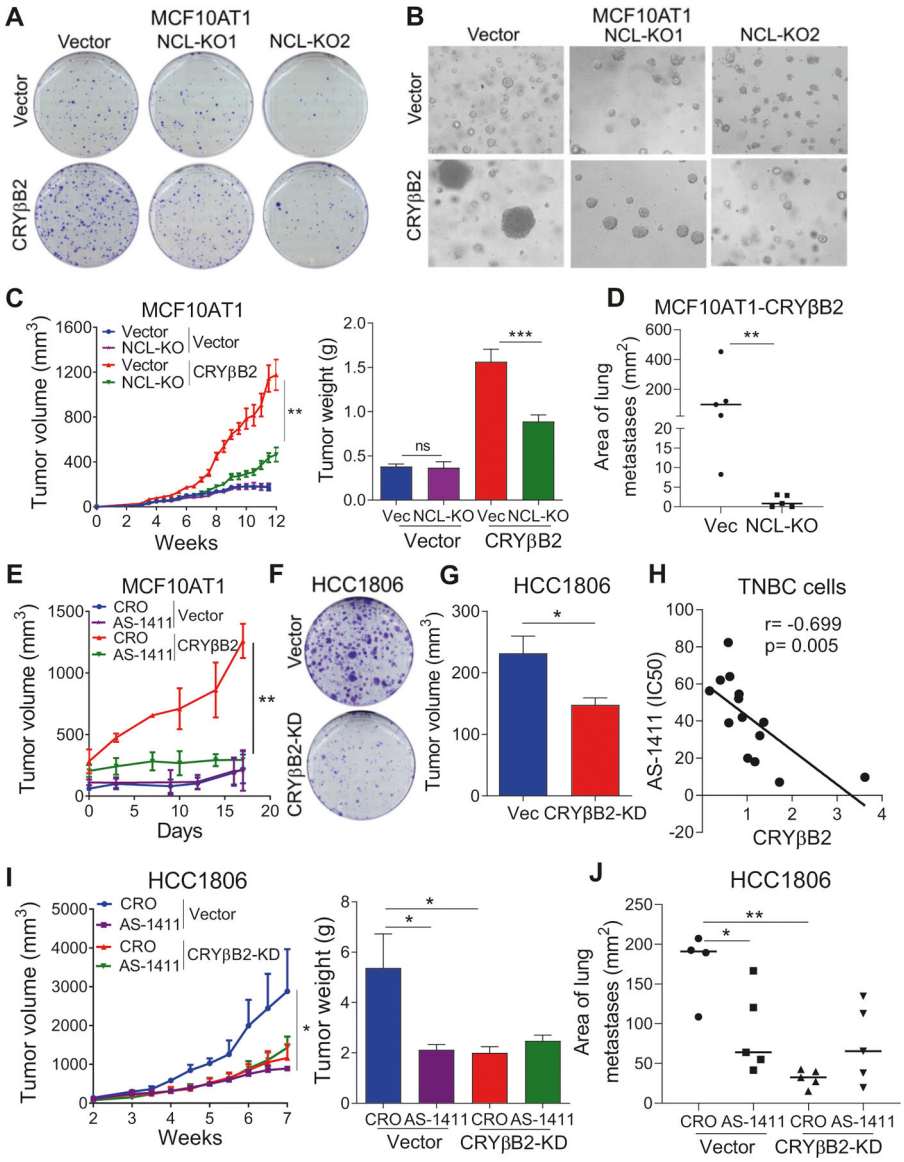

Fig. 5. CRYβB2 tumorigenesis is targeted by nucleolin inhibitors.

Cell proliferation (**A**) and sphere assay (**B**) in triplicate with MCF10AT1-CRYβB2 and control cells with or without nucleolin knockouts (NCL-KO#1 and #2). **C** Tumor volume and weight±SEM and representative tumor images of 5 mice per group injected with MCF10AT1- CRYβB2 and control cells overexpressing vector or NCL-KO. **D** ImageJ quantification of their lung metastases. **E** Tumor volume of 5 mice per group injected with MCF10AT1- CRYβB2 and control cells and treated with the nucleolin aptamer AS-1411 and control CRO (22 μg, i.p). **F** Cell proliferation in the TNBC HCC1806- control and CRYβB2-knockdown (KD) cells. **G**. Tumor volume of 10 mice following 2 weeks of injection with HCC1806- control and CRYβB2- KD cells. **H** Direct correlation of AS-1411 IC50 and CRYβB2 protein expression in TNBC cell lines, determined by MTT proliferation assay and Western blot, respectively. Pearson correlation coefficient (*r*) and the *p*-value are shown. **I** Tumor volume and weight of 5 mice injected with HCC1806- control and CRYβB2- KD

cells and treated with AS-1411 and CRO (22 μg, i.p). **J** ImageJ quantification of their lung metastases. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

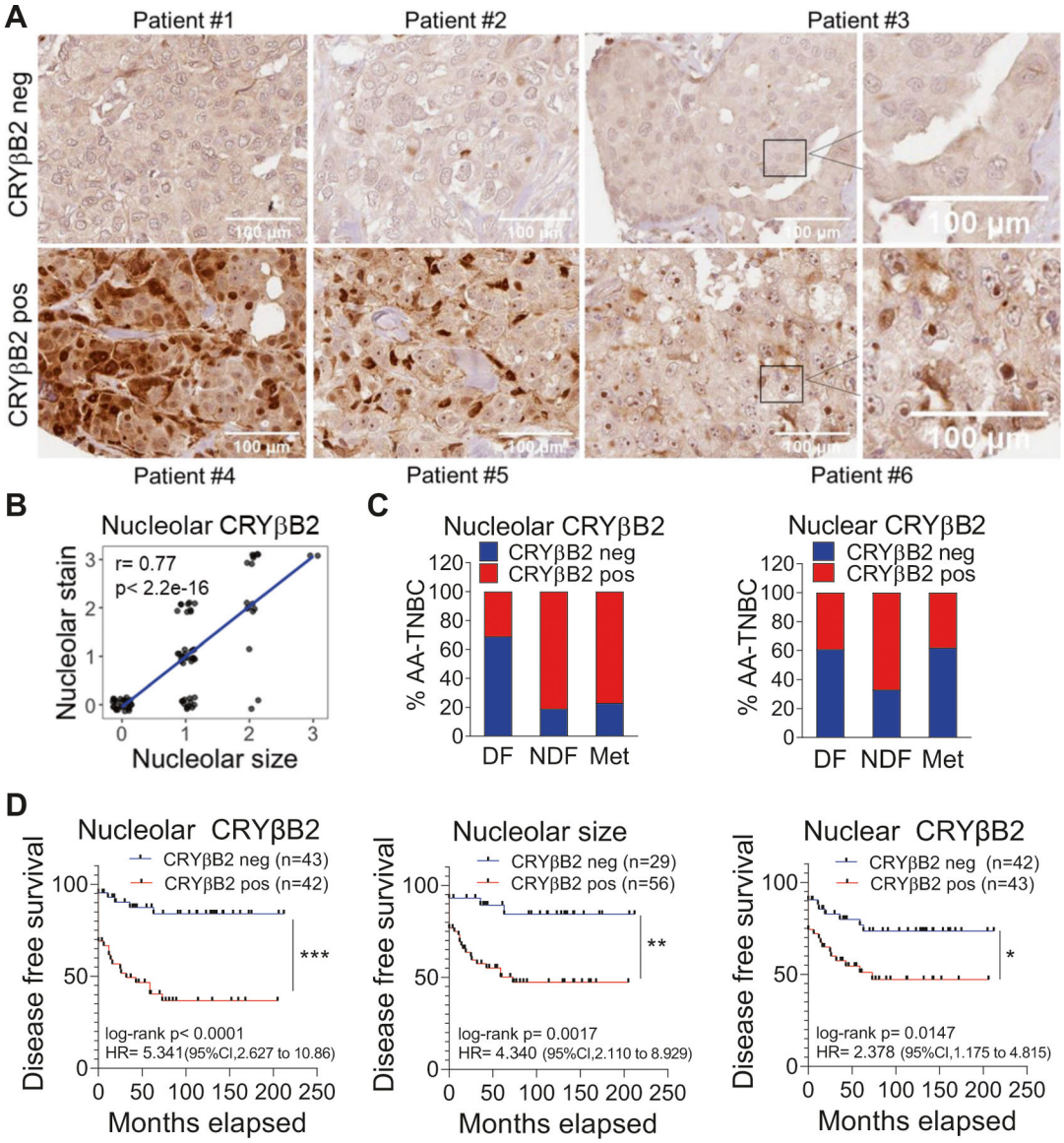

Fig. 6. CRYβB2 associates with poor TNBC outcome in AA women.

A Representative images of CRYβB2 IHC showing CRYβB2 negative and positive TNBC from AA women (n = 102). **B** Correlation of nucleolar CRYβB2 staining with nucleolar size (score 0–3). Pearson correlation coefficient (r) and the p-value are shown. **C** Distribution of tumors with nucleolar and nuclear CRYβB2 negative and positive stain among AA-TNBC patients. DF Disease-free, NDF Never disease-free, and Met Metastatic. **D** Kaplan–Meier plots of disease-free survival (DFS) and log-rank test among AA-patients (n = 85) with TNBC according to the intensity of the staining of CRYβB2 in the nucleolus and its size (positive: score 1–3; negative: score 0) and nucleus (positive: score 2–3; negative: score 0–1).