Impact of germline and somatic cell cycle checkpoint kinase mutations on breast cancer presentation and prognosis.

Seker S[1,2], Punturi NB[1,2], Bainbridge MN[3], Haricharan S[1,2]*.

Affiliations:

[1] Tumor Microenvironment and Cancer Immunology, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA
[2] NCI-designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA
[3] Rady Children's Hospital Genomic Institute of Medicine, San Diego, CA

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*Correspondence to be addressed to Svasti Haricharan (sharicharan@sbpdiscovery.org)

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Abstract

Cell cycle checkpoint kinases activated by DNA repair pathways, ATM/Chk2 and ATR/Chk1 are important tumor suppressors. Germline mutations in these genes associate with increased cancer incidence. Specifically, *ATM* and *CHEK2* germline variants associate with the incidence of estrogen receptor (ER)+ breast cancer with poor patient outcome. More recent investigations identified somatic inactivation of ATM/Chk2 as causal to endocrine therapy resistance in ER+ breast cancer patients. However, the relative contributions of germline and somatic inactivation of these cell cycle checkpoint kinases to ER+/HER2- and ER-/PR-/HER2- or triple negative breast cancer (TNBC) incidence and tumor characteristics has not been systematically assessed. Here, we comprehensively compare the association of *ATM/CHEK2* and *ATR/CHEK1* germline and somatic mutations with age, tumor stage and PR status at diagnosis, and metastatic potential using a meta-dataset compiled from six independent primary and metastatic patient datasets. We observe significantly higher frequency of *ATM* (*~29.6%) and *CHEK2* (9.5%) mutations in metastatic ER+/HER2- breast cancers relative to TNBC (*~11% and 5% respectively)(p=0.03). *CHEK2* mutations associate with higher PR positivity, higher tumor stage and younger age at diagnosis for metastatic ER+/HER2- breast cancer patients. These associations are primarily driven by germline, rather than somatic *CHEK2* mutations. However, somatic CHEK2 mutations associate with more rapid disease progression on early rounds of endocrine therapy. These results provide the first systematic analysis of the contribution of germline and somatic cell cycle checkpoint kinase mutations to tumor characteristics affecting patient prognosis and treatment outcome. Results of this study suggest more streamlined use of the status of these checkpoint kinases as prognostic and/or predictive biomarkers for ER+/HER2- breast cancer patients.

Background

ATM/Chk2 and ATR/Chk1 are cell cycle checkpoints activated by DNA damage as well as many other stressors. Although in reality the pathways are extremely complex, a simplified model is that ATM/Chk2 and ATR/Chk1 inhibit the cell cycle at G1/S and G2/M phases, respectively, to allow the cell time to repair damaged DNA before proceeding with the cell cycle[1]. In cases where prolonged cell cycle arrest is not sufficient to facilitate DNA repair, these checkpoint kinases trigger cell death through both p53-dependent and p53-independent mechanisms[1].

Breast cancer is one of the most commonly diagnosed cancer types in the USA and globally. Estrogen receptor (ER) status of breast cancer dichotomizes breast cancer diagnoses as ER+ and ER-. ER positivity predicts response to endocrine therapies that inhibit ER signaling and are highly effective initially. However, eventually ~40% of ER^+ breast cancer patients become resistant to endocrine therapy[1–3]. Predictive biomarkers for endocrine therapy resistance are a significant clinical need. ER^-, and specifically tumors negative for ER, progresterone receptor (PR) and receptor tyrosine kinase growth factor HER2 (also known as triple negative breast cancer or TNBC) are the more aggressive subtype of breast cancer and have poor patient outcome[4].

We recently uncovered a role for somatic inactivation of ATM/Chk2 signaling through loss of upstream DNA repair pathways that are required to activate ATM/Chk2 in $ER^+/HER2^-$ breast cancer cells and patient tumors[14]. A role for germline variants in *ATM/CHEK2* in ER^+ breast cancer initiation and outcomes has been substantiated by large and multiple independent epidemiological studies[2–11]. Mutations in *CHEK2* and *ATM* associate specifically with increased incidence of ER^+ breast cancer. ER^+ breast cancer patients with *CHEK2* mutations have worse survival outcomes[3,6,12]. Two recent studies independently demonstrated significant association between levels of nuclear pATM and response to endocrine therapy in ER^+ patient tumors[7,13]. However, while germline *ATM/CHEK2* variants have been extensively studied in epidemiological study, there are few studies investigating the role of somatic inactivation of these genes on breast cancer incidence or prognosis. The few studies that have been published have conflicting or inconclusive results[15,16].

Similarly, previous studies of the association of *ATR/CHEK1* mutations, either germline or somatic with breast cancer incidence or prognosis are also inconclusive. Germline mutations in *ATR*, for instance, are observed in familial breast cancer, but whether they contribute substantially to tumor incidence or any specific tumor subtypes remains uncertain[17,18]. However, there appears to be a role for *ATR/CHEK1* somatic dysregulation in triple negative breast cancers (TNBCs)[19–21].

Overall, these cell cycle checkpoint kinases are important guardians of the genome, and well-established tumor suppressors. Understanding their relative contribution to different breast cancer subtypes and prognostic/predictive features is critical for identifying their potential as prognostic/predictive biomarkers as well as therapeutic targets. Here, we undertake a systematic evaluation of the relative contribution of germline and somatic mutations in all four cell cycle checkpoint kinase genes to $ER^+/HER2^-$ and TNBC incidence and tumor characteristics, as described below.

Materials and Methods

Datasets

MSKCC, Cancer Cell 2019 [3], is composed of clinical and mutational data (*ESR1,TP53,ATM,ATR,CHEK2* and *CHEK1* mutations) collected from 1756 patients with hormone receptor positive (i.e. ER and/or PR+) HR+/HER2- (n=1365),HR-/HER2+(n=58), TNBC (n=168) and HR+/HER2+(n=165) primary and metastatic breast cancers. The data is published in 2019.

TCGA, Nature 2012 [5], was downloaded from cBioPortal[6] for clinical and mutational (ESR1,TP53,ATM,ATR,CHEK2 and CHEK1 mutations) analysis in December 2019.The data set is composed of clinical and mutational data collected from 825 patients with HR+/HER2- (n=486),HR-/HER2+(n=31), TNBC (n=123) and HR+/HER2+(n=79) and undetermined (n=106) primary breast cancers.

METABRIC Nature 2012 [7] *& Nat Commun 2016* [8], is downloaded from cBioPortal for clinical and mutational (ESR1,TP53,ATR and CHEK2) analysis in December 2019.The data set is composed of clinical and mutational data collected from 2509 patients with HR+/HER2- (n=1398),HR-/HER2+(n=139), TNBC (n=320) and HR+/HER2+(n=79108) and undetermined (n=544) primary breast cancers.

Broad, Nature 2012 [9], was downloaded from cBioPortal for clinical and mutational (ESR1,TP53,ATM,ATR,CHEK2 and CHEK1 mutations) analysis in March 2020.The data set is composed of clinical and mutational data collected from 825 patients with HR+/HER2- (n=37),HR-/HER2+(n=6), TNBC (n=320) and HR+/HER2+(n=108) and undetermined (n=544) primary breast cancers.

MBCP, Provisional, February 2020 [10], was downloaded from cBioPortal for clinical and mutational (*ESR1,TP53,ATM,ATR,CHEK2* and *CHEK1* mutations) analysis in March 2020.The data set is composed of clinical and mutational data collected from 825 patients with HR+/HER2- (n=50),HR-/HER2+(n=8), TNBC (n=8) and HR+/HER2+(n=21) and undetermined (n=93) metastatic breast cancers.

British Columbia, Nature 2012 [11], was downloaded from cBioPortal for clinical and mutational (*ESR1,TP53,ATM,ATR,CHEK2* and *CHEK1* mutations) analysis in March 2020.The data set is composed of clinical and mutational data collected from 825 patients with HR+/HER2- (n=9), TNBC (n=90) and undetermined (n=8) primary breast cancers.

Mutational analysis

For mutation analysis, *ATM, CHEK2, ATR* and *CHEK1* mutations were studied and *TP53* and *ESR1* mutations were used as controls. All non-synonymous mutations were included irrespective of category (i.e. missense, nonsense, frameshift, etc) or predicted pathogenicity. Mutational data were compared in three main categories: Receptor status (ER^+ vs TN), Sample site (primary, PRI vs metastatic, MET) and Mutation Type (Germline vs Somatic). Mutational frequency was calculated based on total number of mutations identified in a category and the total patient size of the same category. Samples without any of the indicated mutations are labeled as wildtype (WT).

Tumor characteristics

PR status, tumor stage and age of diagnosis of the patients were used as categorical variables to determine patient sample characteristics with respect to mutational data. Fisher's exact test determined p-values by comparing different categories such as $ER^+/HER2^-$ vs TN or Germline vs Somatic for PR status and tumor stage while Two-tailed Wilcoxon rank sum tests were used for continuous age differences.

Survival and Disease Progression analysis

For univariate analyses, all tumors with associated survival data, is obtained from MSKCC data set. Outcome measures used were progression-free survival and progression on endocrine treatment. Only samples with survival metadata were included in the analysis.

Statistical analysis

Missing data were imputed with "NA" from mutation and survival data analysis. Samples classifying for more than one category were treated as separate set for statistical comparisons. Two-tailed Wilcoxon rank sum tests were used for age comparisons and Pearson's Chi Square test (or Fisher's Exact test) was used for comparing categorical data. Log rank test calculated p-values for survival analyses and Cox regression determined proportional hazards.

Results

ATM/CHEK2 mutations are more frequent in ER+ breast cancer while TNBC is predisposed to ATR mutations

Because mutations in cell cycle checkpoint kinase genes are relatively rare in breast cancer, we created a meta-dataset using six independent datasets to ensure sufficient sample size of ER^+ primary, ER^+ metastatic and TNBC (**Figure 1**). Using this meta-dataset, we first analyzed the incidence of mutations (germline or somatic) in each of the four cell cycle checkpoint kinases, *ATM, CHEK2, ATR, CHEK1*), in ER^+ vs TNBC samples. We included mutations in *ESR1* and *TP53* as positive controls (known drivers) for ER^+ and TNBC respectively. As expected, we found a statistically significant increase in frequency of *ESR1* mutations in ER^+ breast cancer and of *TP53* mutations in TNBC samples (**Figure 2A-B**). Overall, we observed comparable frequency of mutations in the cell cycle kinase genes between ER^+ and TNBC (**Figure 2A-B**).

However, when we considered mutational frequency of each cell cycle checkpoint kinase gene, we observed a statistically significant enrichment for *ATM* and *CHEK2* ($p=0.02$) mutations in ER^+ breast cancer samples but no enrichment for either *ATR* or *CHEK1* mutations (**Figure 2C-D**). Incidence of mutations in *CHEK1* was extremely rare in either ER^+ or TNBC. Of note, while mutations in any of these checkpoint kinases co-occurred with mutations in *TP53* in TNBC, this was almost never the case in ER^+ breast cancer (**Figure 2C-D**), likely due to the significantly higher incidence of *TP53* mutations in TNBC. These data suggest a bifurcation in the importance of ATM/Chk2 and ATR/Chk1 pathways to ER^+ and TNBC.

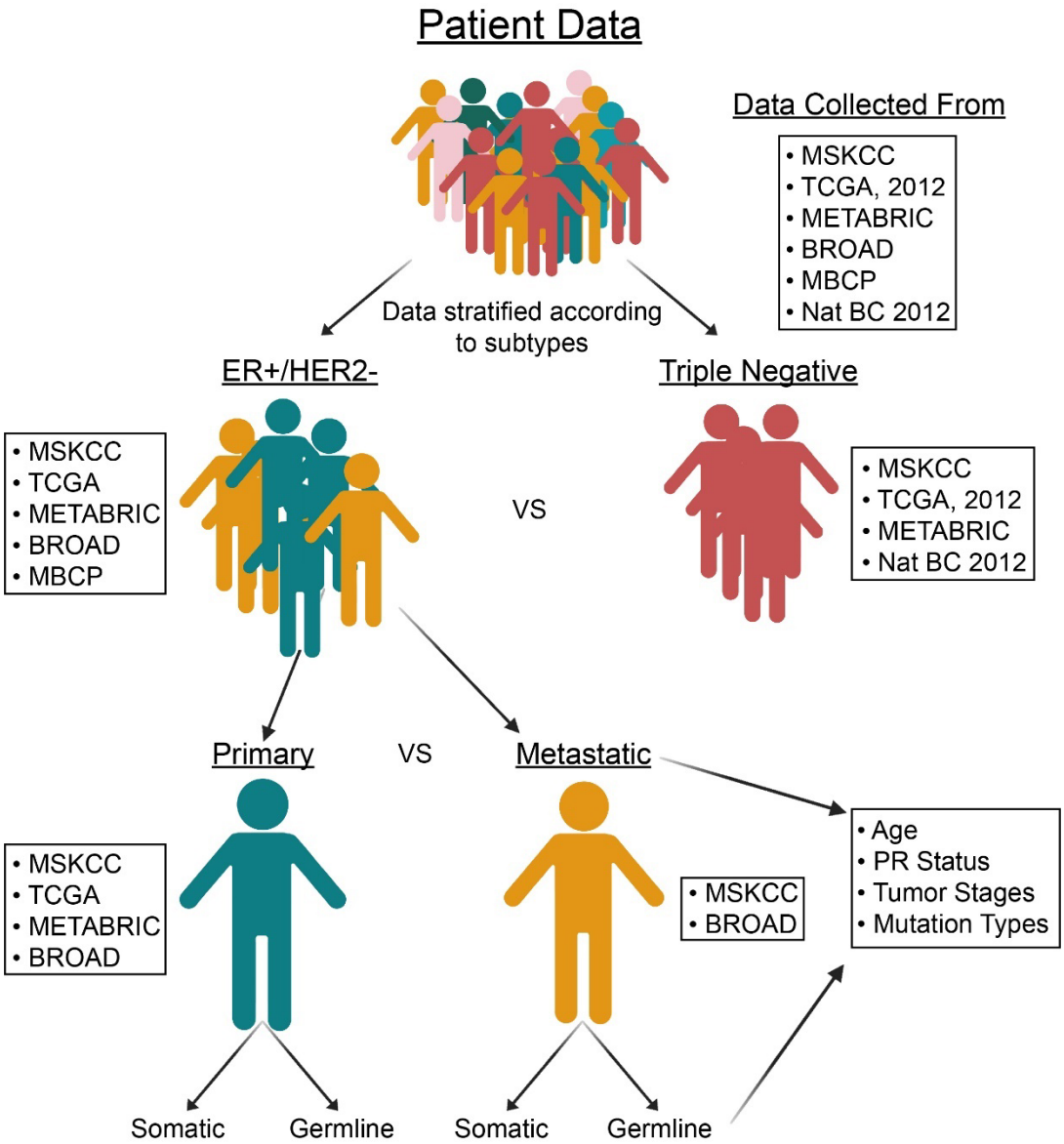

Figure 1: Outline of study. Data collected from: MSKCC, Cancer Cell 2019, TCGA Nature 2012, METABRIC, Nature 2012 & Nat Commun 2016, Broad, Nature 2012, MBCP, Provisional, February 2020, and British Columbia, Nature 2012. Patient data stratified and analyzed based on molecular subtype of disease, sample type, and occurrence of mutations (Germline Vs. Somatic). Data was further analyzed in the context of age of patient, PR status, tumor stage and mutation type.

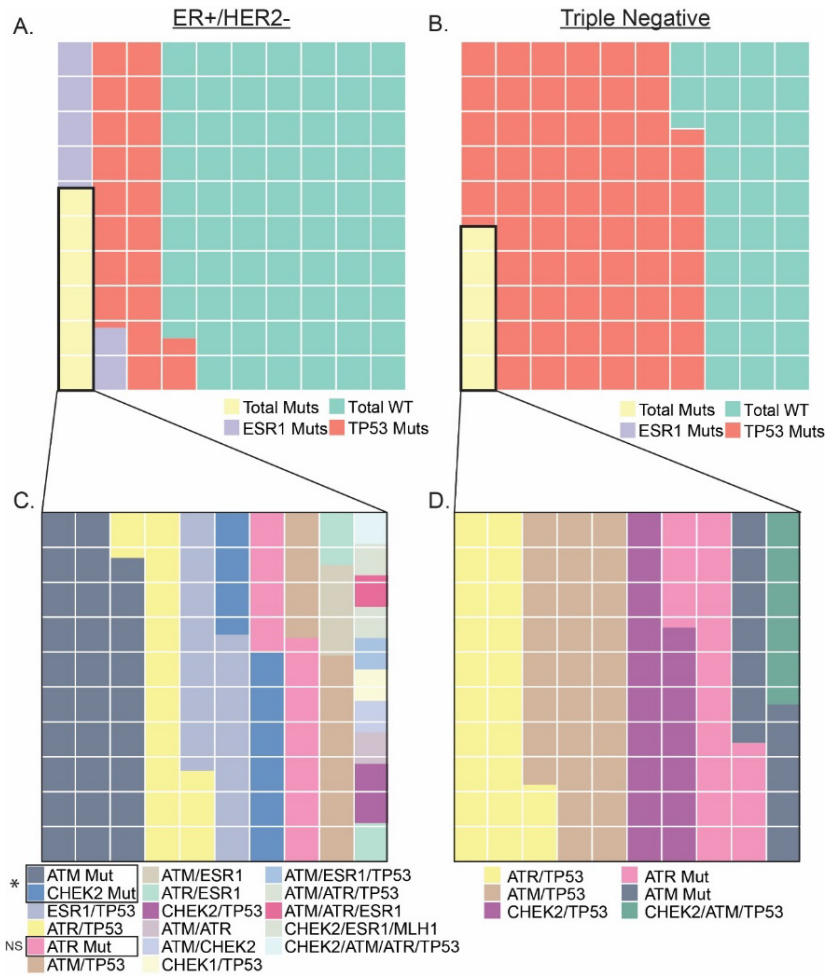

Figure 2. Mutational frequency of cell cycle checkpoint kinase genes based on breast cancer subtype. (A-B) Waffle chart charts showing *ESR1, TP53*, and cell cycle checkpoint kinase mutational frequencies in ER$^+$/HER2$^-$ vs TNBC and (C-D) frequency distribution of cell cycle checkpoint kinase mutations in ER$^+$/HER2$^-$ vs TNBC. Each square represents 1% of overall mutations in specified genes totaling 100% of our genes of interest. Fisher's Exact test determined p-values. $p<0.05$*, ns = not significant, WT, wildtype, Mut, mutant.

We next tested whether frequency of mutations in any cell cycle checkpoint kinase gene (except *CHEK1*, which is rarely mutated in ER$^+$ breast cancer) was enriched in metastatic ER$^+$ breast cancer. We found significant increases in mutational frequency of *ATR* and *CHEK2* in metastatic ER$^+$ breast cancer relative to either primary ER$^+$ or TNBC, similar to *ESR1*, but not *TP53* (**Figure 3A-E, Figure S3**). Incidence pattern of mutations in these genes was highly comparable to that of *ESR1* mutations, a known driver of metastatic ER$^+$ breast cancer[22–25] (**Figure 3A**), and dissimilar to *TP53*, which is more frequently mutated in TNBC than either primary or metastatic ER$^+$ breast cancer (**Figure 3B**). We also assessed the landscape of mutations in *ATM* and *CHEK2*, the two cell cycle checkpoint kinase genes most frequently mutated in ER+ breast cancer, between primary and metastatic samples. The overall mutational profile was comparable between primary tumors and metastases for both genes, with roughly 30% of mutations being deleterious

(frameshift or nonsense) and the majority being missense (**Figure 3F-G**). Furthermore, we observed no detectable differences in mutation clusters in any specific protein domains. Overall, these data suggest a role for *CHEK2* mutation in metastatic ER+ breast cancer.

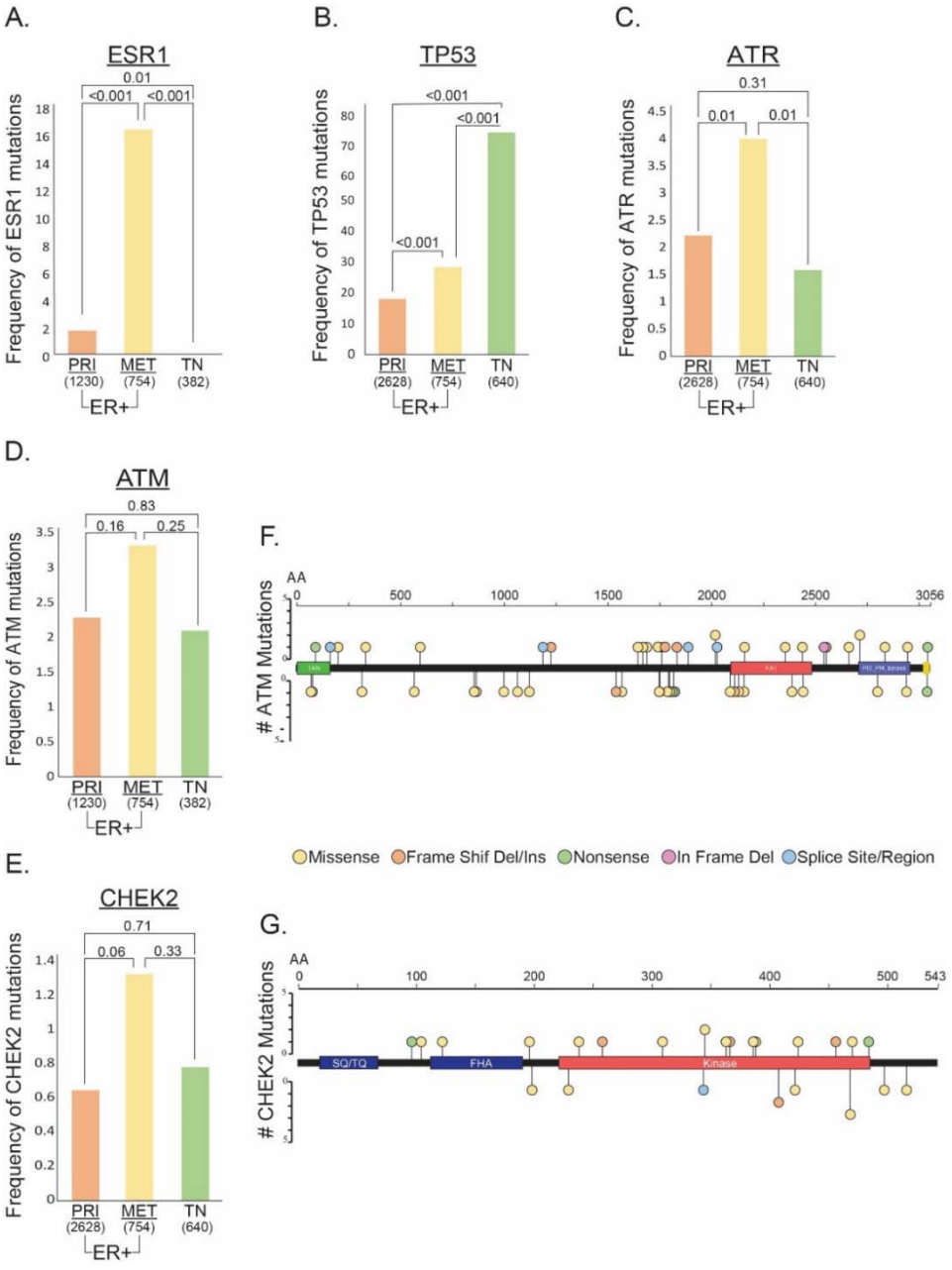

Figure 3. Mutational frequency of cell cycle checkpoint kinase genes based on tumor type (primary or metastatic). (A – E) Bar graphs representing frequency of *ESR1, TP53, ATR, ATM,* and *CHEK2* mutations in $ER^{+}/HER2^{-}$ primary versus metastatic and in TNBC. TNBC column is composed of both primary and metastatic samples. (F-G) Lolliplots of *ATM* and *CHEK2* mutations categorized by

amino acid location and mutation type. Mutations found in primary samples are represented below the gene domain depiction while mutations in metastatic samples are represented above it.

***CHEK2* mutations associate with higher PR positivity and younger age at diagnosis in metastatic ER⁺ breast cancer**. We next tested whether *ATM* or *CHEK2* mutations associated with known prognostic factors in either primary or metastatic ER⁺ breast cancer samples. We found that *CHEK2* mutations had an association pattern that was comparable to that of *ESR1* mutations for all tumor characteristics assessed. Mutations in *CHEK2*, like mutations in *ESR1*, associated with significantly higher PR positivity in metastatic ER⁺ breast cancer (**Figure 4A**), supporting a role for these genes in regulating oncogenic ER signaling. We also observed no differences in tumor stage at diagnosis in primary or metastatic ER⁺ tumors with mutations in any of the genes analyzed, although wildtype tumors presented with significantly higher stage at diagnosis if the cancer was metastatic (**Figure 4B**). Also, *CHEK2*, but not *ATM*, mutations associate with a significantly younger age at diagnosis of metastatic ER⁺ breast cancer patients (**Figure 4C**), similar to *ESR1*. These differences were confirmed within MSKCC, the largest dataset in our meta-dataset that had both primary and metastatic samples (**Figure S3**).

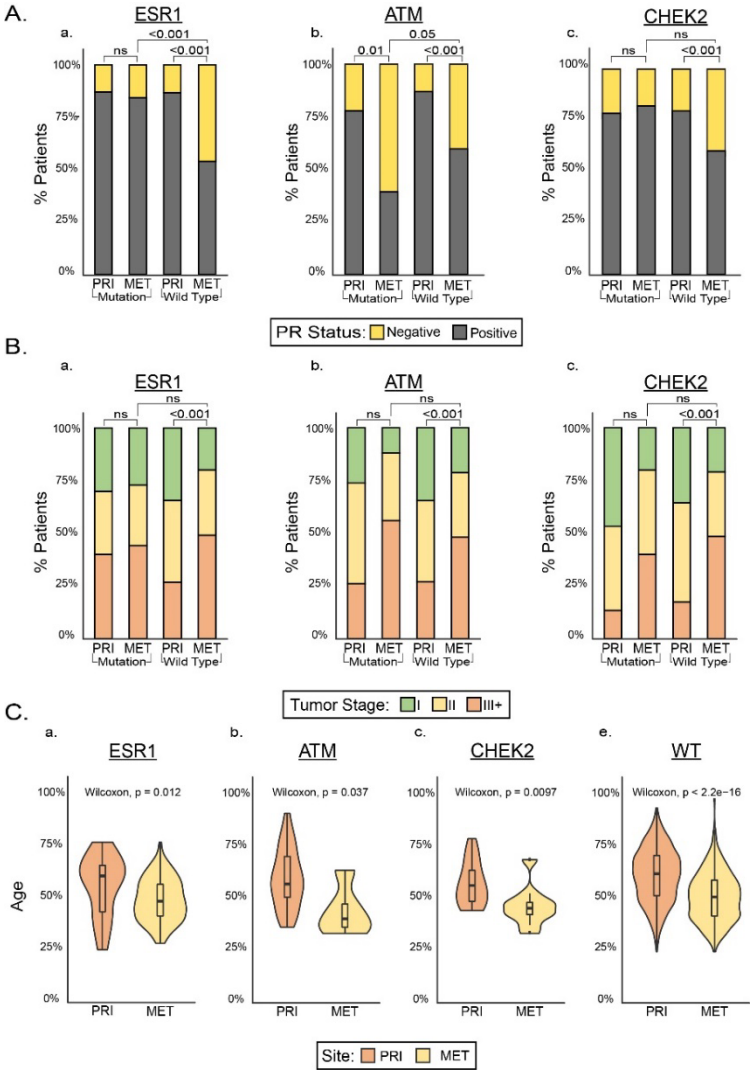

Figure 4. Comparison of primary versus metastatic samples based on PR status, tumor stage, and age at diagnosis in ER$^+$/HER2- disease. (A&B) Stacked columns graphs representing PR status (A) and tumor stage (B) at diagnosis of primary vs metastatic ER$^+$ breast cancer patients with mutations in any of the specific genes. Frequencies are compared for statistical significance to wildtype tumors lacking mutations in any of these genes, using Fisher's Exact test. (C) Violin plots representing age at diagnosis of primary vs metastatic ER$^+$ breast cancer patients whose tumors either had mutations in *ATM*, *CHEK2* or *ESR1* or were WT.

This younger age at diagnosis could be primarily driven by germline, rather than somatic *CHEK2* mutations. Therefore, we parsed our meta-dataset to separate out germline and somatic mutations to assess their individual associations with these known tumor characteristics. As expected, we observed no germline *ESR1* mutations (**Figure 5A**). Interestingly, we found germline *ATM* mutations only in primary ER$^+$ breast cancer samples (**Figure 5B**), while we found enrichment for germline *CHEK2* mutations in metastatic ER$^+$ breast cancer (**Figure 5C**, p=0.01). We compared the landscape of germline *CHEK2* mutations to somatic ones and found a similar number of missense vs deleterious (nonsense, frameshift or splice site) mutations in both groups (**Figure 5D**). However, we found increased incidence of deemed pathogenic mutations in the germline vs somatic called variants, likely due to differences in call filtering (**Figure 5D**). These data suggest that *CHEK2* germline mutations are more likely to induce metastatic ER$^+$ breast cancer at a younger age, while germline mutations in *ATM* associate with more benign primary ER$^+$ breast cancer incidence.

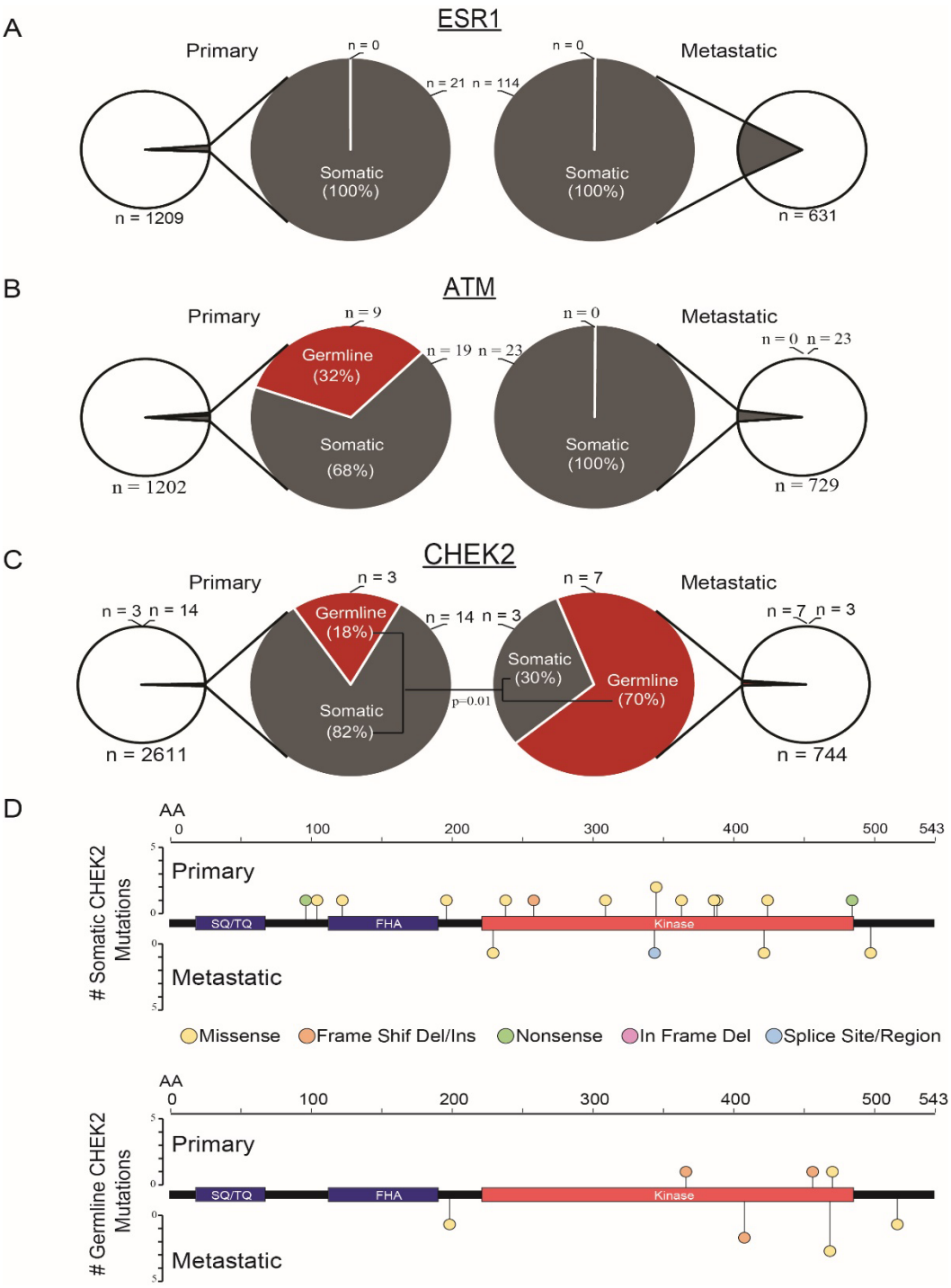

Figure 5. Percentage of germline vs somatic mutations in *ATM* and *CHEK2*. (A-C) Pie charts representing proportion of mutations in each specific gene, *ESR1* (A), *ATM* (B) and *CHEK2* (C) in ER^+ primary and metastatic samples, with an expanded pie of the proportion of germline vs somatic mutations for each gene. (D) Lolliplots of CHEK2 germline and somatic mutations in primary vs metastatic ER^+ breast cancer samples. Fisher's Exact test determined p-values.

***CHEK2* germline mutations drive associations with higher PR positivity and younger age at diagnosis in metastatic ER+ breast cancer**

To identify individual contributions of germline and somatic *CHEK2* mutations to the various tumor characteristics analyzed, we assessed whether missense and deleterious somatic mutations in *CHEK2* compared to germline mutations in terms of PR positivity, tumor stage and age at diagnosis in primary and metastatic ER$^+$ breast cancer. We found that somatic mutations in *CHEK2* associated with higher PR negativity, similar to *CHEK2* wildtype status, in ER+ metastatic samples, but not in primary samples (**Figure 6A**).

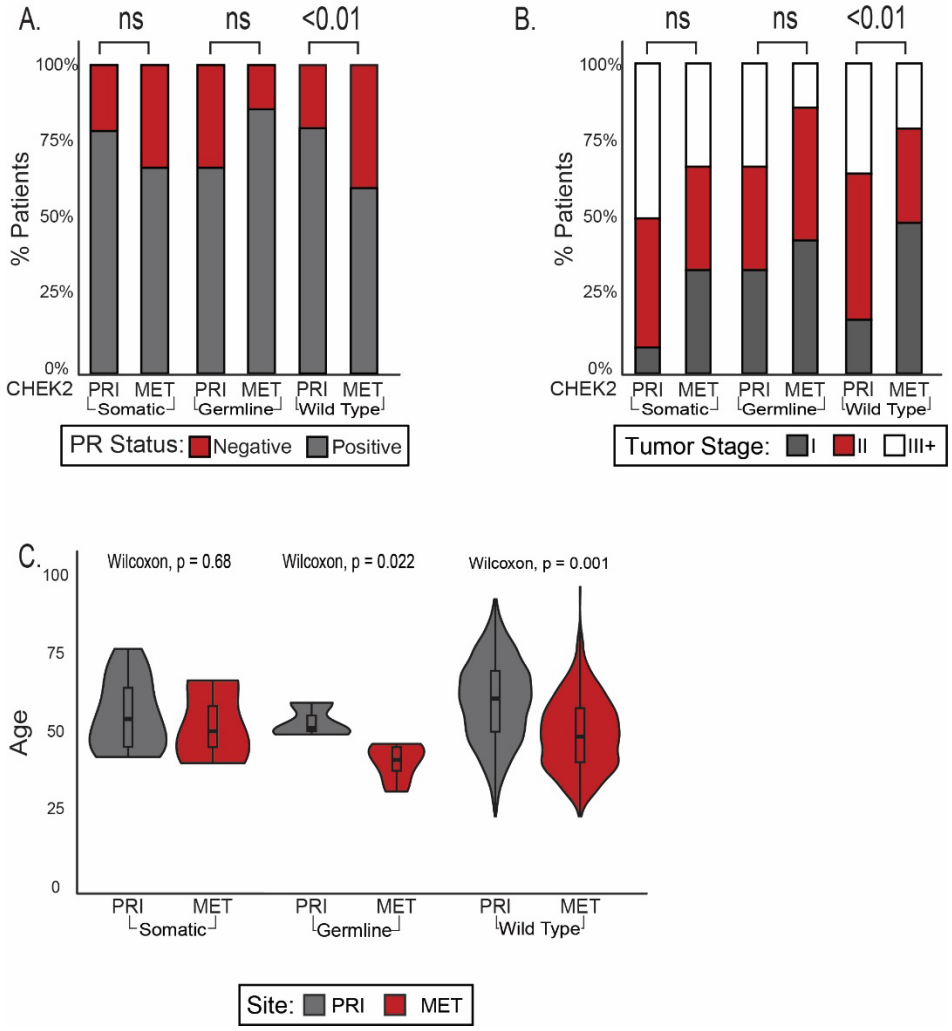

Figure 6. Association of germline vs somatic mutations in *CHEK2* with known prognostic tumor characteristics. Stacked columns graphs representing PR status (A) and tumor stage (B) at diagnosis of primary vs metastatic ER$^+$ breast cancer patients with germline vs somatic mutations in *CHEK2*. Frequencies are compared for statistical significance to wildtype tumors lacking mutations in any of these genes, using Fisher's Exact test. (C) Violin plots representing age at diagnosis of primary vs metastatic ER$^+$ breast cancer patients whose tumors either had germline or somatic mutations in *CHEK2* or were WT.

Similarly, we found that while germline mutations in *CHEK2* associated with high tumor stage irrespective of metastatic status, somatic mutations in *CHEK2*, similar to wildtype

tumors, had lower tumor stage at diagnosis in primary samples relative to metastatic ones (**Figure 6B**). Finally, we found that the younger age at diagnosis for metastatic ER^+ breast cancer patients was driven by germline, rather than somatic, mutations in *CHEK2* (**Figure 6C**). These differences remain statistically significant in a within-dataset comparison of MSKCC patient data (**Figure S4**).

Deleterious mutations in any cell cycle checkpoint kinase gene **associates with worse progression-free survival on endocrine treatment**

We next tested whether mutations in cell cycle checkpoint kinase genes associate with worse overall survival in the MSKCC dataset. We did not have sufficient numbers of mutated tumors in the primary setting, so we restricted our analysis to metastatic ER^+ breast cancer patients. While we found no significant association between mutations in any of the genes and disease-free or overall survival (**Figure S5**), we found significant association with deleterious *ATM* and *CHEK2* mutations and worse progression-free survival on endocrine treatment in metastatic ER^+ breast cancer patients (**Figure 7A-B**). Specifically, we found a splice site mutation in *CHEK2* and a frameshift mutation in *ATM* as associating significantly with progression on endocrine treatment (**Figure 7A-B**). No *ATR* mutation associated with progression free survival (**Figure 7C**).

To analyze this further, we assessed time to progression on each endocrine treatment for patient with mutations in *CHEK2*. In general, patients were administered on average, 4 consecutive endocrine therapies, with 10 years being the maximum duration on each. The maximum number of endocrine therapies administered was nineteen. We found that somatic mutations in *CHEK2* associated with more rapid progression beginning with the very first endocrine treatment offered (**Figure 7D**), while germline mutations in *CHEK2* associated with more gradual progression, although still more rapid than wildtype tumors (**Figure 7E**). Overall, these data provide further evidence of a unique and robust association between Chk2 inactivation, whether germline or somatic, and endocrine therapy response.

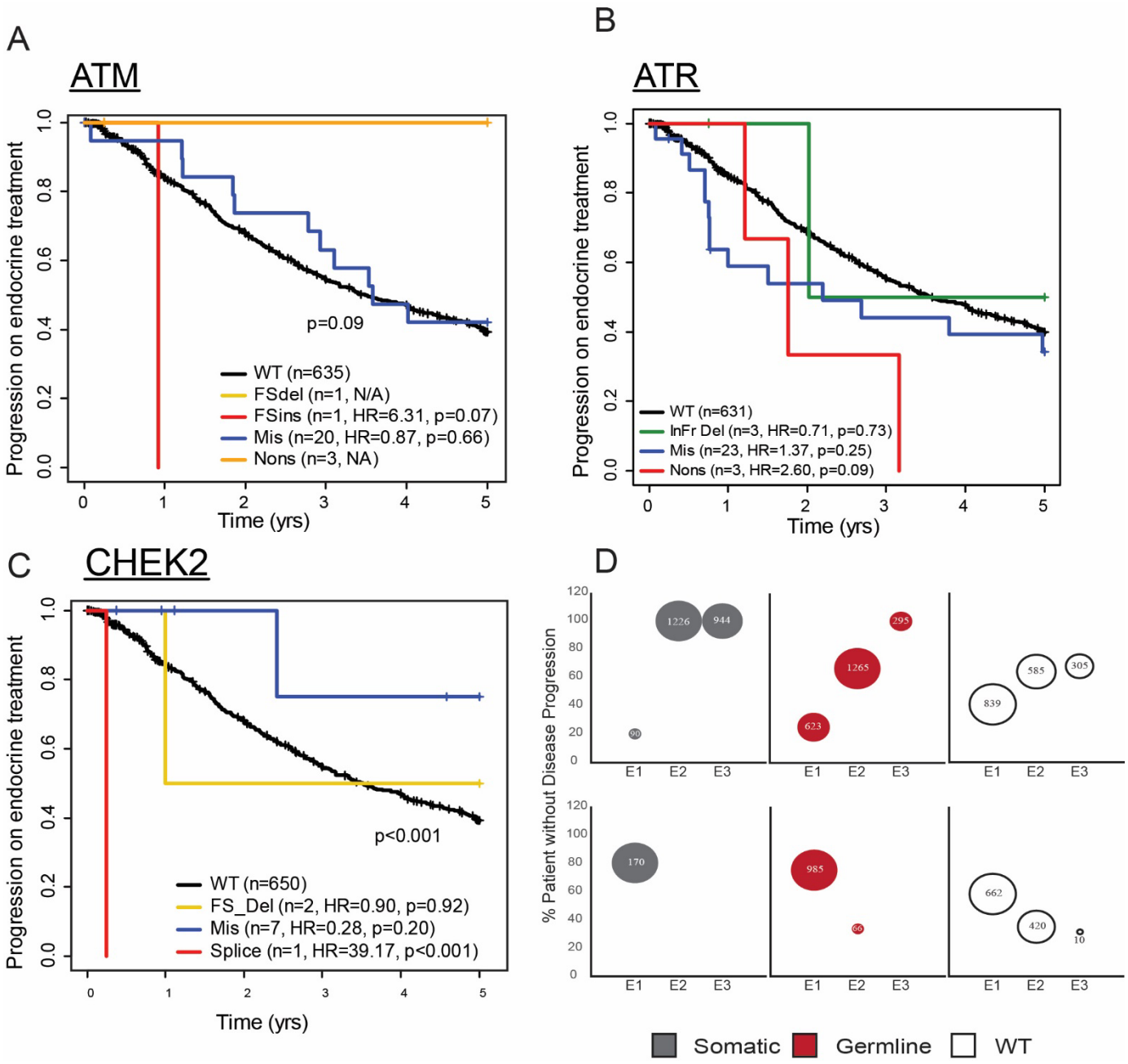

Figure 7. Association of cell cycle checkpoint kinase gene mutations with patient outcome. (A-C) Kaplan-Meier survival curves of progression-free survival on endocrine treatment of metastatic ER+ breast cancer patients. Log rank test determined p-values. (D) Bubble plots depicting duration of time on each of the first four endocrine treatment regimens of metastatic ER+ breast cancer patients from MSKCC, with the size of the bubble depicting the length of time, and each plot depicting %

patients who progressed or did not progress on each endocrine treatment on the y-axis. Associated data in **Figure S5**.

Discussion

A role for cell cycle checkpoint kinases as tumor suppressors in many cancers, including breast is well-established[1]. Moreover, a distinction between ATM/CHK2 and ATR/CHK1 signaling in terms of cell cycle regulation has been well documented[26–28]. Generally, ATM/Chk2 appears integral to single strand break repair and G1/S cell cycle regulation[14,27,29], while ATR/Chk1 is activated by double strand break repair pathways and regulates G2/M[19,21]. Our systematic analysis of these two cell cycle signaling nodes across ER^+ and TNBC identifies a dichotomy where ATM/Chk2 associates with ER^+ breast cancer. These data suggest a division in terms of DNA repair between these two cancer types, which is reflected in the differential impact of these two cell cycle signaling nodes in ER^+ vs TNBC. A fundamental recharacterization of all breast cancer based on cell cycle signaling nodes, and DNA repair proficiencies might constitute a therapeutically valid system of characterization, as these cell cycle signaling nodes can translate to specific CDK dependencies, which can induce sensitivity to specific CDK inhibitors[30].

Germline variants in *ATM* and *CHEK2* are known to predispose women to ER^+ breast cancer. We further demonstrate that germline variants in these genes associate with younger age at diagnosis of metastatic ER^+ breast cancer, and higher tumor stage at diagnosis even in terms of primary ER^+ breast cancer, both poor prognosticators. Interestingly, we find that *CHEK2* germline variants associate with increased PR positivity in the metastatic ER^+ breast cancer setting. This is a curious finding, since PR positivity generally associates with better outcome. These data suggest more complex mechanistic underpinnings between Chk2 and ER signaling that require experimental investigation.

The role of somatic inactivation of ATM and Chk2 in ER^+ breast cancer outcome has remained conflicted, with few reported publications. We recently identified a role for lack of activation of ATM/Chk2 in endocrine therapy resistance, induced by somatic loss of DNA repair proteins[14,31]. Results described here suggest that somatic mutations in *CHEK2* may also play a role in defining tumor characteristics of primary ER^+ breast cancer. A specific role for such mutations in treatment response remains to be tested.

Overall, the systematic study of ATM and ATR signaling node mutation described here is the first to parse germline and somatic events in the context of primary and metastatic ER^+ breast cancer, as well as TNBC. The results of this study suggest a broader role for ATM/Chk2 in ER^+ breast cancer, and perhaps, a similarly important role for ATR in TNBC. There is potential for the development of prognostic and predictive biomarkers based on the status of these cell cycle checkpoint kinases, as well as avenues for development of CDK inhibitors that can be matched to each tumor's cell cycle dependencies.

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