



## Invited Review

# Hormonally up-regulated neu-associated kinase: A novel target for breast cancer progression



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## ABSTRACT

Hormonally up-regulated neu-associated Kinase (Hunk) is a protein kinase that was originally identified in the murine mammary gland and has been shown to be highly expressed in Human Epidermal Growth Factor Receptor 2 positive (HER2<sup>+</sup>/ErbB2<sup>+</sup>) breast cancer cell lines as well as MMTV-neu derived mammary tumor cell lines. However, the physiological role of Hunk has been largely elusive since its identification. Though Hunk is predicted to be a Serine/Threonine (Ser/Thr) protein kinase with homology to the SNF1/AMPK family of protein kinases, there are no known Hunk substrates that have been identified to date. Recent work demonstrates a role for Hunk in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer progression, including drug resistance to HER2/ErbB2 inhibitors, with Hunk potentially acting downstream of HER2/ErbB2 and the PI3K/Akt pathway. These studies have collectively shown that Hunk plays a vital role in promoting mammary tumorigenesis, as Hunk knockdown via shRNA in xenograft tumor models or crossing MMTV-neu or *Pten*-deficient genetically engineered mouse models into a Hunk knockout (*Hunk*<sup>-/-</sup>) background impairs mammary tumor growth in vivo. Because the majority of HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer patients acquire drug resistance to HER2/ErbB2 inhibitors, the characterization of novel drug targets like Hunk that have the potential to simultaneously suppress tumorigenesis and potentially enhance efficacy of current therapeutics is an important facet of drug development. Therefore, work aimed at uncovering specific regulatory functions for Hunk that could contribute to this protein kinase's role in both tumorigenesis and drug resistance will be informative. This review focuses on what is currently known about this under-studied protein kinase, and how targeting Hunk may prove to be a potential therapeutic target for the treatment of breast cancer.

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**Abbreviations:** Hunk, hormonally up-regulated neu-associated kinase; EGFR/HER1/ErbB1, epidermal growth factor receptor; HER2<sup>+</sup>/ErbB2<sup>+</sup>, human epidermal growth factor receptor 2 positive; MMTV, mouse mammary tumor virus; PI3K, phosphoinositide 3-kinase; Pten, phosphatase and tensin homolog.

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## 1. Introduction

Hormonally Up-regulated Neu-associated Kinase (Hunk) is a Serine (Ser)/Threonine (Thr) protein kinase that was discovered in a screen to identify protein kinases expressed in the murine mammary gland during development and in mammary epithelial tumor cells derived from MMTV-neu, MMTV-wnt (int2), MMTV-myc, and MMTV-ras genetically engineered mouse models [1]. In the same year that Hunk was identified in the mammary gland, the mouse and human homologs of this protein kinase were cloned and the human gene was mapped to chromosome 21q22.11 [1–3]. Hunk is an ~80 kDa protein encoded by the *Hunk* gene containing multiple regions of homology to members of the SNF1/AMPK protein kinase family, including a catalytic kinase domain that is C-terminally flanked by a small region designated the SNF1 homology domain (SNH domain) [2–4]. The SNF1/AMPK family of protein kinases has known roles in metabolism, proliferation, differentiation, survival, migration, and invasion, and are expressed in many types of cancers [4,5]. However, whether Hunk functions in these processes is relatively unknown.

In addition to the SNH domain, Hunk has a ubiquitin-associated domain (UBA domain) located downstream of its kinase region, which is also seen in many AMPK protein kinases [6]. In typical AMPK family members, this domain is not used to bind ubiquitin but is necessary for kinase activation and conformational stability [7]. A distinction in Hunk is that the catalytic domain contains a stretch of positively charged amino acids resembling the PIP2 consensus binding site, which is not found in other AMPK-related protein kinases [6]. PIP2 is a major phospholipid component of the plasma membrane that has various functions, such as the regulation of processes requiring membrane reorganization, including endocytosis [8]. Hunk also contains a unique C-terminal domain that is not homologous to any known protein to date [9]. It is possible that these domain distinctions will prove to be essential for Hunk intracellular functions.

In the literature, Hunk is also known as metastasis associated protein kinase in VMR tumor family (MAK-V) [2]. Hunk cDNA is 98% homologous to MAK-V cDNA, with only one codon altered between the two, though the different codons encode the same amino acid resulting in the same protein structure for Hunk and MAK-V [2]. Therefore, it was determined that both the *Hunk* gene and *MAK-V* gene are one in the same [2]. Hunk chromosomal location at 21q22.11 shows close proximity to the *SOD1* gene, a factor implicated in the pathology of amyotrophic lateral sclerosis (Lou Gehrig's disease). Because Hunk was found to be expressed in several areas of the brain and throughout the central nervous system, it was originally thought that Hunk may be implicated in nervous system malignancies [6], though this has not been confirmed.

In addition to being expressed in the mammary gland, brain, and CNS, *Hunk* has also been found to be transcriptionally expressed in many other tissues including the ovary, liver, kidney, thymus, and lung [2,3,9,10]. It is important to note that despite the evidence of *Hunk* expression in these tissues, transcriptional expression is typically found within specific subsets of cells rather than having a global distribution. Early reports on Hunk from Gardner et al. aimed to determine Hunk's potential for regulation of embryonic development, since *Hunk* was found to be transcriptionally expressed in embryonic cells [3]. The authors of this study showed that in the developing murine embryo, *Hunk* is highly expressed during mid-gestation, but decreases in some tissues before birth, indicating a temporal regulation of *Hunk* expression during development. These early studies were the first to suggest a tissue-specific developmental role for Hunk, paving the way for investigation into its regulation of mammary gland development. However, Hunk is not essential for normal development, as studies in *Hunk*<sup>-/-</sup> mice indicate that loss of *Hunk* does not impair embryonic viability [4]. Despite a

wide tissue distribution of *Hunk* expression, much of what has been studied on Hunk's molecular functions has been focused on breast tumor pathogenesis.

## 2. Intracellular functions of Hunk

To date, the intracellular functions of Hunk have remained somewhat elusive. Though Hunk's kinase domain has high homology to the AMPK family of protein kinases with specificity for Ser/Thr residues, neither a consensus phosphorylation sequence nor bona fide substrates have been identified for Hunk. Instead, most of the experiments evaluating Hunk protein kinase activity have been directed at determining whether a catalytically-inactive mutant form of Hunk alters protein localization or protein-protein interactions. Studies investigating the requirement of Hunk kinase activity will be further discussed below.

In turn, since protein kinases are often subject to phosphorylation by other protein kinases that regulate downstream activity, one study investigated Hunk as a target for phosphorylation in COS-7 cells. This group suggested that Hunk may be phosphorylated by unknown protein kinases specifically at Ser residues, having not seen phosphorylation on any Thr residues [11]. To identify potential phosphorylation sites on Hunk, additional studies using quantitative mass spectrometry-based analyses with serial enrichments of different post-translational modifications (SEPTM) were implemented to identify Hunk phosphorylation sites at S65, S360, S368, Y378, S561, S585, and T618 [12]. Consequently, further investigation into these specific Hunk phosphorylation sites to identify the protein kinase(s) that target these sites in Hunk, as well as the outcome of Hunk function due to phosphorylation at each site, is warranted.

Early studies assessed Hunk's subcellular localization in various mammalian cell types and showed Hunk to be localized in the cytoplasm, at the plasma membrane, as well as in the nucleus [6,13,14]. One study by Kalinichenko et al. showed that Hunk was largely associated with membrane fractions in neuronal cells, murine-derived mammary tumor cells, and in yeast [6]. It was also demonstrated through the use of Hunk-deletion mutants in a yeast-two-hybrid assay that the Hunk UBA domain was required for Hunk membrane localization [6]. This study by Kalinichenko et al. suggested that the UBA domain was necessary for Hunk to bind the plasma membrane component PIP2 thereby localizing Hunk to the membrane [6]. Interestingly, when exogenously expressed, wild-type Hunk was found to be in the nucleus and at centrosomes as well [6,14]. Furthermore, the localization of wild-type Hunk to these intracellular regions was found to a greater extent than a kinase-dead mutant of Hunk in several different cell lines [6,14]. These reports were some of the earliest indications that the kinase activity of Hunk regulates this protein's intracellular localization, which is predicted to effect Hunk's intracellular functions. Centrosome localization suggests Hunk may be involved in regulation of the cell cycle, possibly during mitosis, and that these functions require Hunk kinase activity, though this has not been proven [14].

Surprisingly few binding partners of Hunk have been identified. The Nedd4 E3 ubiquitin ligase was shown to be a binding protein of Hunk in PC12 cells, which was determined by mass spectrometry using Hunk isolated from PC12 cells expressing a doxycycline-inducible Hunk. The binding of Hunk to Nedd4 was confirmed by co-immunoprecipitation of Nedd4 with endogenous Hunk in CSML-0 cells [15]. Proteins that bind to Nedd4 typically interact through proline-rich PY motifs [16–18]. However, Hunk does not contain a PY-motif, suggesting a unique avenue of interaction [15]. The interaction between Hunk and Nedd4 was shown to be dependent on the presence of the amino-terminal domain of Hunk containing the kinase domain. This study by Kalinichenko et al.

also demonstrated that Hunk is a target of proteasome-dependent degradation, and that Hunk is ubiquitinated independently of Nedd4, as Nedd4 inhibition does not alter Hunk stability [15]. In addition to Nedd4, an interaction between Hunk and synaptopodin has also been identified [19]. This study, also performed by Kalinichenko et al. determined synaptopodin bound to recombinant Hunk through its C-terminal domain using mass spectrometry [19]. Because synaptopodin has known roles in dendritic spine contact and synaptic plasticity [20,21], it was suggested by the authors that this interaction may lead to future identification of Hunk function in the nervous system.

One of the earliest roles suggested for Hunk was a potential involvement in the regulation of endocytosis. This idea stemmed from studies by Korobko et al. who showed using yeast-two hybrid that Hunk interacts with Rabaptin-5, an effector of the GTPase Rab5, a protein that regulates endocytosis and membrane trafficking through early endosome fusion [2,22]. Overexpression of wild-type Hunk also showed an increase in fluid-phase endocytosis. This effect was not as dramatic when kinase-deficient Hunk was overexpressed, implying Hunk may have a kinase-dependent regulatory role in endocytosis [2]. The authors further suggested that Hunk might phosphorylate essential endocytic proteins since the intact kinase domain of Hunk was required for the observed increase in endocytosis [2]. However, phosphorylation was not tested and further studies will be required to determine if any endocytic proteins are Hunk substrates.

Additional studies have aimed to elucidate a role for Hunk in growth factor signaling. A study by Komurov et al. found that Hunk promotes EGFR (a.k.a. HER1/ErbB1) signaling upon EGF ligand binding in a kinome screen that investigated protein kinases which become active in response to EGF treatment [23]. Another publication by Yeh et al. showed that Hunk is regulated in response to EGF stimulation where EGF treatment of a HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cell line resulted in an increase in Hunk protein levels [24]. It is currently unknown if EGFR can independently regulate Hunk or if this effect on Hunk expression is due to the dimerization between HER2/ErbB2 and EGFR. Most recently, it has been reported that Hunk knockdown is able to inhibit EGFR activity indicated by reduced EGFR phosphorylation in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells resistant to HER2/ErbB2 inhibitors [25], which could suggest that Hunk participates in a positive feedback loop to regulate EGFR activation.

Most notably, the SNF1/AMPK family of protein kinases regulate stress-induced metabolic changes [26]. Recent work indicates that Hunk regulates autophagy in a manner that promotes tumor cell survival in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells, thereby acting as a mechanism for drug resistance [13]. Autophagy is a stress-induced process essential for the maintenance of intracellular homeostasis that results in the degradation of cellular materials enclosed within a double-membraned vesicle, the autophagosome [27,28]. The study by Yeh et al. suggested Hunk may regulate autophagy, as Hunk-deficient mammary gland fibroblasts and HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells expressing Hunk shRNA show reduced autophagic flux [13]. Furthermore, Hunk knockdown inhibited autophagy induced by HER2/ErbB2 inhibitor treatment in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells [13]. Likewise, targeting Hunk by shRNA downregulation in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells obtained from a patient who was resistant to HER2/ErbB2 inhibitors reduced autophagy [13]. These findings suggest Hunk may be regulating autophagy in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells as a survival mechanism in a manner that contributes to acquisition of therapeutic drug resistance. Additional studies suggest that Hunk regulates cell survival during adult mammary gland development as well as mammary tumor cell survival via apoptosis [29]. Similarly, Hunk expression was able to promote viability in pre-differentiated neurons [10]. Whether the effect of Hunk on cell survival due to

autophagy is related to the effect of Hunk on apoptosis remains to be determined as significant crosstalk exists between proteins that regulates these two processes.

Since SNF1/AMPK-like protein kinases have been shown to regulate proliferation, it is also possible that Hunk regulates proliferation in addition to affecting cellular survival. In one such study, Sakai et al. evaluated how Hunk functions in renal tubular cells and showed that Hunk overexpression suppressed angiotensin II (ANGII) [9]. The resulting effect was an inhibition of proliferation due to the suppression of regulatory proteins that promote proliferation, such as c-fos and TGF- $\beta$ . These findings imply that Hunk may negatively regulate proliferation, though this may be tissue and context dependent. An additional study indicating Hunk negatively regulates proliferation was analyzed in intestinal cells [30]. In this study, the authors show that in the normal intestine, Hunk negatively regulates proliferation, as Hunk deficiency resulted in increased proliferation and migration [30]. In addition to the kidney and intestine, Hunk overexpression was also shown to reduce proliferation of alveolar epithelial cells during pregnancy in the mammary epithelium of MMTV-*Hunk* transgenic mice [31]. However, a more recent study showed Hunk to positively regulate proliferation in breast and mammary tumor cells, suggesting context-dependent and cell-specific proliferative functions of Hunk [24].

### 3. Hunk regulates mammary gland development

One of the earliest studies on Hunk focused on its role in murine mammary gland development induced by changes during pregnancy [31]. In this study, Gardner et al. found that Hunk is upregulated in a subset of epithelial cells in the mammary gland during early pregnancy or when oophorectomized mice are stimulated with 17 $\beta$ -estradiol and progesterone treatment [31]. This was the first paper to suggest that Hunk may be regulated by ovarian hormones in pregnancy. These observations are thought to extend to include breast cancer, as estrogen and progesterone have an established role in the etiology of breast tumorigenesis [31,32], although the effect of these hormones on Hunk in breast cancer has never been evaluated. Interestingly, transgenic Hunk overexpression in MMTV-*Hunk* mice caused impaired lobuloalveolar development and subsequent defects in lactation, with decreased epithelial proliferation and differentiation at later stages of pregnancy, suggesting a time-dependent regulatory role of Hunk for pregnancy-induced mammary gland changes [31]. Since estrogen and progesterone are required for epithelial proliferation in puberty and alveolar differentiation in pregnancy respectively, it is possible that these hormones regulate Hunk downstream to aid in estrogen-induced proliferation in the mammary gland. However, later studies in *Hunk*<sup>-/-</sup> mice did not report changes in proliferation during pregnancy [4,29].

Further work investigating Hunk's role in mammary gland development by Yeh et al. evaluated apoptosis in the involuting mammary gland and found that mammary glands from *Hunk*<sup>-/-</sup> mice during involution showed significantly more apoptotic activity, suggesting Hunk is a promoter of cell survival in the mammary gland during adult mammary gland development [29]. The authors determined that Hunk was needed to maintain cell survival during mammary gland involution via down-regulation of the apoptotic molecule myc, downstream of Akt [29]. These findings led the authors to further investigate whether Hunk promoted tumor cell survival in response to Akt activation in a mammary specific *Pten*-conditional knockout genetically engineered mouse model through a similar mechanism tied to myc expression levels [29]. As expected, they found that Hunk was necessary for the survival of cells in mammary tumors driven by loss of the tumor suppress-

sor *Pten* in vivo, as Hunk-deficient tumors showed significantly more apoptosis [29]. Additional experiments showed that upon Akt activation, Hunk is up-regulated and consequently suppresses c-myc expression [29,33]. Taken together, these findings indicate that Hunk is an Akt effector with the ability to suppress myc-induced apoptosis.

#### 4. Hunk and breast cancer metastasis

Hunk has also been shown to have a role in metastasis. But, whether Hunk is a metastasis promoting factor or a metastasis inhibiting factor is not clear. An initial report by Wertheim et al. showed in a MMTV-*myc* mammary tumor model bred into a *Hunk*<sup>-/-</sup> background that although Hunk was dispensable for mammary tumor formation induced by the *myc* protooncogene, it was required for the metastasis of these tumors to the lung, since MMTV-*myc* mice containing wild-type *Hunk* readily metastasized in comparison to mice in the *Hunk*<sup>-/-</sup> background [4]. This study also showed that the kinase-activity of Hunk was required for myc-induced mammary tumor metastasis, as MMTV-*myc*; *Hunk*<sup>-/-</sup> derived mammary tumor cells expressing a kinase-dead mutant of Hunk showed significantly fewer lung metastases in an in vivo metastasis assay, compared with those cells expressing a wild-type version of Hunk [4]. In vitro migration assays confirmed the in vivo observations on metastasis as Hunk kinase activity was also found to be required for myc-induced mammary tumor migration and invasion [4].

Although some studies on Hunk have shown Hunk to be a promoter of metastasis, one study strongly suggests otherwise [34]. A study by Quintela-Fandino et al. concluded that Hunk suppresses metastasis of basal-like breast cancers [34]. The study authors used human breast cancer cell lines classified as basal-like to overexpress wild-type Hunk in order to show that Hunk impaired metastasis via a cofilin-dependent mechanism, where Hunk is thought to inhibit cofilin-mediated actin filament formation by sustaining cofilin phosphorylation [34]. However, the phosphorylation of cofilin was not shown to be directly mediated by Hunk but rather through PP2A interaction and presumably PP2A phosphatase activity toward cofilin [34]. Interestingly, later studies also showed that Hunk does not suppress or induce metastasis in luminal or basal-like tumors but in genetically engineered mouse models [24,29]. Since the role of Hunk cannot clearly be ruled in favor of promoting or inhibiting metastasis, further characterization of Hunk-mediated metastasis is warranted to determine its functions in different breast cancer subtypes.

#### 5. Hunk is implicated in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer and development of drug resistance

In 2004, four years after Hunk was cloned, Korobko et al. reported Hunk to be overproduced in about 50% of breast carcinomas with tumor cells staining positive for Hunk displaying primarily cytoplasmic localization of the protein [35]. However, the observed increase in Hunk levels in this study did not correlate with histological subtype including hormone receptor status (estrogen and progesterone), metastasis, or an increase in proliferative markers. Interestingly, increased levels of Hunk were more frequently observed in HER2<sup>+</sup>/ErbB2<sup>+</sup> (3+) tumor samples compared to HER2<sup>-</sup>/ErbB2<sup>-</sup> [35]. Separate genomic analysis of human breast tumor tissues in a later study confirmed a potential association of higher *Hunk* expression in some human breast cancers, particularly correlating with the HER2<sup>+</sup>/ErbB2<sup>+</sup> subtype [4]. Consistent with prior reports, we have now performed an additional distinct analysis using gene expression data from The Cancer Genome Atlas (TCGA) and found that HER2<sup>+</sup>/ErbB2<sup>+</sup> breast

cancer samples have higher *Hunk* expression levels than that of estrogen receptor negative (ER<sup>-</sup>)/HER2<sup>-</sup> and estrogen receptor positive (ER<sup>+</sup>)/HER2<sup>-</sup> breast cancers (Fig. 1A, adjusted  $p < 0.001$  and  $p < 0.001$ , respectively). It is also worth noting that there was no observed difference in *Hunk* expression in this analysis between ER<sup>-</sup>/HER2<sup>-</sup> and ER<sup>+</sup>/HER2<sup>-</sup> groups, despite the potential regulation of *Hunk* by estrogen as previously described [31].

Perhaps not surprisingly, based on the analyses described above, much of the scientific research on Hunk has focused on evaluating Hunk's role in breast cancer etiology and progression with particular focus on the HER2<sup>+</sup>/ErbB2<sup>+</sup> subtype. Multiple lines of investigation demonstrated that targeting Hunk, either by shRNA knockdown in MMTV-*neu*-derived mammary tumor cells and HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells, or through the use of MMTV-*neu*-genetically engineered mouse models bred into a *Hunk*<sup>-/-</sup> background, significantly impaired tumor growth [13,24,25]. It was also reported that a kinase-dead mutant of Hunk impaired tumor growth [24], suggesting Hunk kinase activity is important for the promotion of HER2<sup>+</sup>/ErbB2<sup>+</sup> mammary tumorigenesis in vivo.

Mechanistically, Hunk was shown to be upregulated in response to HER2/ErbB2 oncogene activation and targeting Hunk induced apoptosis in response to stabilization of p27<sup>kip1</sup>, a cyclin-dependent kinase inhibitor and tumor suppressor [24]. HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancers downregulate p27<sup>kip1</sup> as a mechanism to promote tumor cell proliferation and survival whereas upregulation and subsequent stabilization of p27 leads to cell cycle arrest and cell death [36]. As already noted, studies by Yeh et al. suggest Hunk also plays a mechanistic role in regulating autophagy in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells as a potential mechanism of cell survival [13]. However, the specific pathway or pathways, such as regulation of p27 that Hunk signals within to regulate autophagy in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer has yet to be determined.

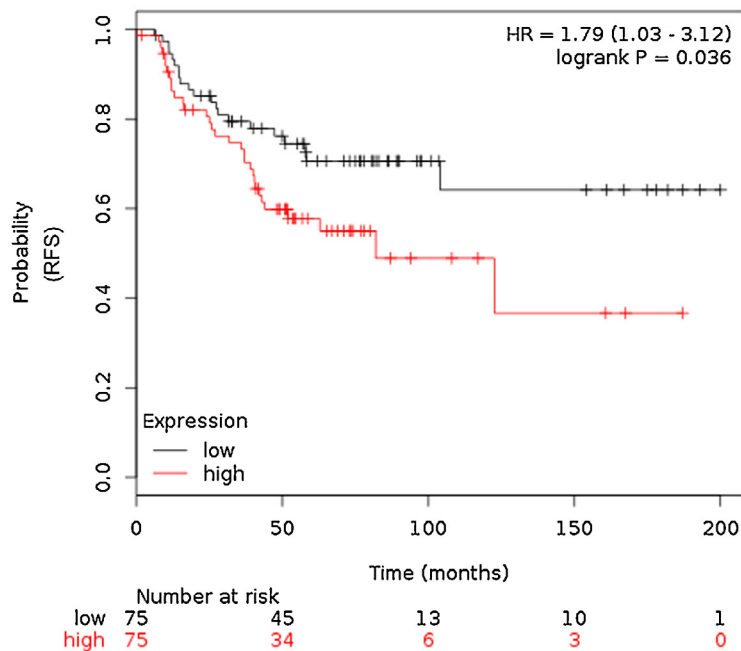
The most recent studies on Hunk implicate this protein kinase in the development of drug resistance to HER2/ErbB2 inhibitors in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer. Two recent studies demonstrated that targeting Hunk with shRNA in a HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cell line derived from a patient who was resistant to trastuzumab impaired mammary tumor growth in vivo [13,25]. These studies provided evidence to suggest that Hunk regulates the catabolic process of autophagy, which can lead breast cancer cells to become resistant to targeted inhibitors, and that downregulating Hunk by shRNA inhibits autophagy in both primary and drug resistant HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells. It is currently understood that autophagy plays a dual role in cancer, displaying both tumor-suppressing and tumor-promoting properties in a context-dependent manner [28,37,38]. During early tumor formation, autophagy is considered to be tumor suppressive [37]. However, in established HER2<sup>+</sup>/ErbB2<sup>+</sup> tumors, autophagy is regarded as a pro-survival mechanism which allows cancer cells to overcome stressful environments such as hypoxia or increased metabolic demands [27]. Furthermore, many chemotherapeutic agents and targeted agents, including HER2/ErbB2 inhibitors, induce autophagy [28,39,40]. In response to these observations, several clinical trials are currently aimed at assessing the effect of pharmacologic inhibition of autophagy in order to determine if inhibiting this process improves the efficacy of chemotherapeutic agents [37]. Because HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells that have become resistant to HER2/ErbB2 inhibitors exhibit higher levels of basal autophagy [39,41,42], it is proposed that Hunk upregulates autophagy in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer cells as a survival mechanism, thus promoting the acquisition of drug resistance to HER2/ErbB2 inhibitors. Therefore, targeting Hunk has the potential to improve the treatment of drug resistant HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancers through downregulation of autophagy.

Additional studies from the same group found that targeting both Hunk and another protein kinase JNK was able to effec-

A

Comparison	n (HER2 pos)	n (HER2 neg)	log2FC (HER2pos/HER2neg)	p-val	adjusted p-val
HER2 pos v HER2 neg	114	648	0.676	2.68E-05	0.00028
Comparison	n (ER pos)	n (ER neg)	log2FC (ERpos/ERneg)	p-val	adjusted p-val
ER pos (HER2-) v ER neg (HER2-)	483	136	0.1843	0.277	0.32
Comparison	n (HER2 x)	n (ER x)	log2FC (HER2x/ERx)	p-val	adjusted p-val
HER2 neg v ER neg	619	31	-0.784	0.00507	0.021
HER2 neg v ER pos	619	79	-0.588	0.00197	0.017
HER2 pos v ER pos	114	483	0.616	0.00015	0.00069
HER2 pos v ER neg	114	136	0.800	0.0003	0.00079

B

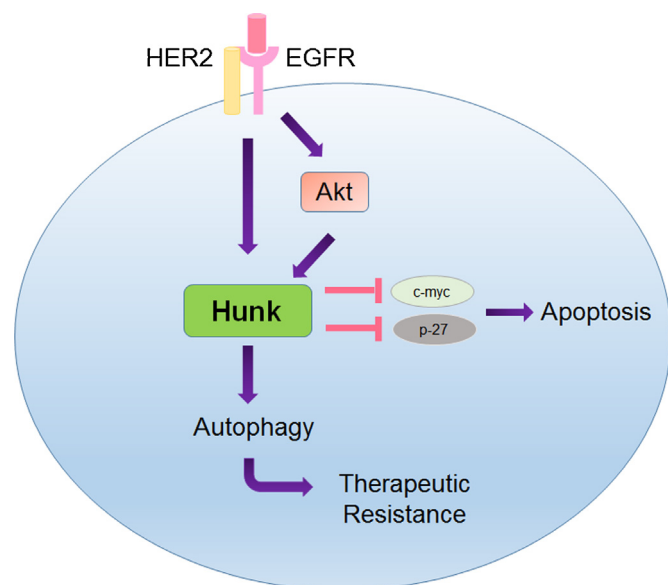


**Fig. 1.** Hunk analysis. A. *Hunk* expression is higher in HER2<sup>+</sup>/ErbB2 breast cancers. Gene expression data (RNAseqV2 RSEM normalized) were retrieved for the TCGA breast invasive carcinoma dataset (BRCA, v2016.01.28) from the BROAD GDAC FireBrowse site. The HER2, estrogen receptor (ER), and progesterone receptor (PR) status was retrieved from the UCSC Cancer Genomics Browser. Pairwise comparisons were made by first generating subsets of samples, followed by removal of genes with few reads using a cutoff that required the sum of counts across the subset to be greater than 20. Voom was used to transform the RSEM normalized counts prior to running a moderated *t*-test on the data [45,46] followed by a Benjamini-Hochberg procedure to correct for multiple testing. Specifically, even though only results for *Hunk* are shown here, expression levels of all 20,533 genes were used for analysis.  $p < 0.0001$ . B. *Hunk* expression is higher in HER2<sup>+</sup>/ErbB2 breast tumors and is associated with reduced recurrence free survival (RFS) in HER2<sup>+</sup>/ErbB2 patients. Gene probe *Hunk* 1555935\_s.at was used for analysis with HER2<sup>+</sup> status set to “positive” yielding  $n = 150$  patient samples with available clinical data containing the selected events. A total of  $n = 75$  patients were scored as “low” *Hunk* and  $n = 75$  were scored as “high” *Hunk*. Analysis tool automatically removed redundant samples and excluded any biased arrays. The probe expression range was classified as 4–578 with a cutoff value of 127 used for analysis. HR = 1.79, logrank  $p$ -value = 0.036.

tively suppress growth of HER2<sup>+</sup>/ErbB2<sup>+</sup> drug resistant tumors in vivo better than individual inhibition of either Hunk or JNK [25]. Because enhancement of signaling by molecules downstream of Epidermal Growth Factor Receptor (EGFR) are often implicated in HER2-inhibitor drug resistance, the authors aimed to determine if combinatory targeting of Hunk with an identified compensatory signaling pathway could suppress tumor growth of drug resistant HER2<sup>+</sup>/ErbB2<sup>+</sup> mammary tumors. The study authors found that combined inhibition of both Hunk and JNK promoted cell death and overall impaired autophagy of HER2-inhibitor resistant HER2<sup>+</sup>/ErbB2<sup>+</sup> cells in vitro, and inhibited tumor growth of HER2<sup>+</sup>/ErbB2<sup>+</sup> drug resistant mammary tumors in vivo [25]. These results corroborate a function for Hunk in HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer drug resistance, and suggest that Hunk inhibition, perhaps in a chemotherapeutic drug cocktail, may be a clinically useful approach in combating drug resistance.

One area of Hunk research that is currently lacking is investigation of Hunk as a biomarker in clinical human breast cancer samples. In order for Hunk to be considered a clinically viable ther-

apeutic target, it is important to know how Hunk expression and activity translates in breast cancer patients. While some studies have been performed to analyze Hunk gene expression in human breast cancer samples and were discussed earlier, additional comprehensive analyses with large patient cohorts are required to drive clinical progress. We have recently performed analysis using the Kaplan-Meier plotter database tool (kmplot.com), which uses gene expression data from Gene Expression Omnibus (GEO), the European Genome-phenome Archive (EGA), and The Cancer Genome Atlas (TCGA), to assess whether *Hunk* gene expression correlates with relapse free survival (RFS) in the HER2<sup>+</sup>/ErbB2<sup>+</sup> breast cancer subtype [43,44]. Our analysis using this online tool indicates that *Hunk* expression correlates with reduced RFS, consistent with previously published evidence (Fig. 1B, *Hunk* probe 1555935\_s.at) [4,35]. While encouraging, these studies are likely a small part of further, much needed analyses of Hunk's role in human breast cancer for Hunk to be considered as a viable therapeutic target.



**Fig. 2.** An overview of Hunk-mediated intracellular signaling in HER2<sup>+</sup>/ErbB2 breast cancer. HER2/ErbB2 activation induces Hunk expression as well as stimulates downstream Akt signaling, which can in turn upregulate Hunk. Hunk is then able to downregulate c-myc in response to Akt activation, or relocate p-27 in response to HER2/ErbB2 activation, ultimately promoting survival. Hunk activation can also promote autophagy, which can then further enhance survival and promote drug resistance.

## 6. Conclusion

In this review, we have attempted to describe what is currently known concerning Hunk's physiological functions, particularly in breast cancer with focus on the HER2<sup>+</sup>/ErbB2<sup>+</sup> subtype. These roles include kinase-dependent cellular functions for Hunk including endocytosis, apoptosis, migration, proliferation, and autophagy (Fig. 2). However, because Hunk substrates have yet to be identified, it is still mechanistically unknown as to how the kinase activity of Hunk is important for these various functions. As more clinically relevant data provides evidence for targeting Hunk in breast cancer, this novel protein kinase will prove to be a clinically viable pharmacological target.

## Conflict of interest

The authors declare they have no competing interests. Identification of certain commercial equipment, instruments, software or materials does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the products identified are necessarily the best available for the purpose.

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JZ and EY conceived and wrote the paper. BN generated the methods and prepared the gene expression analysis presented in Fig. 1A.

## References

- [1] L.A. Chodosh, et al., Protein kinase expression during murine mammary development, *Dev. Biol.* 219 (2) (2000) 259–276.
- [2] I.V. Korobko, E.V. Korobko, S.L. Kiselev, The MAK-V protein kinase regulates endocytosis in mouse, *Mol. Gen. Genet.* 264 (4) (2000) 411–418.
- [3] H.P. Gardner, et al., Cloning and characterization of Hunk: a novel mammalian SNF1-related protein kinase, *Genomics* 63 (1) (2000) 46–59.
- [4] G.B. Wertheim, et al., The Snf1-related kinase: Hunk, is essential for mammary tumor metastasis, *Proc. Natl. Acad. Sci. U. S. A.* 106 (37) (2009) 15855–15860.
- [5] A. Salminen, K. Kaarniranta, A. Kauppinen, AMPK and HIF signaling pathways regulate both longevity and cancer growth: the good news and the bad news about survival mechanisms, *Biogerontology* 17 (August (4)) (2016) 655–680, <http://dx.doi.org/10.1007/s10522-016-9655-7>.
- [6] S.V. Kalinichenko, E.V. Korobko, I.V. Korobko, Membrane localization of the MAK-V protein kinase, *Biochemistry (Mosc.)* 73 (3) (2008) 278–282.
- [7] M. Jaleel, et al., The ubiquitin-associated domain of AMPK-related kinases regulates conformation and LKB1-mediated phosphorylation and activation, *Biochem. J.* 394 (Pt 3) (2006) 545–555.
- [8] N. Gamper, M.S. Shapiro, Target-specific PIP(2) signalling: how might it work? *J. Physiol.* 582 (Pt 3) (2007) 967–975.
- [9] M. Sakai, et al., Expression of MAK-V/Hunk in renal distal tubules and its possible involvement in proliferative suppression, *Am. J. Physiol. Renal. Physiol.* 292 (5) (2007) F1526–F1536.
- [10] I.V. Korobko, et al., Pro-survival activity of the MAK-V protein kinase in PC12 cells, *Cell Cycle* 9 (20) (2010) 4248–4249.
- [11] I.V. Korobko, et al., Phosphorylation of MAK-V protein kinase in mammalian cells, *Dokl. Biochem. Biophys.* 412 (2007) 37–39.
- [12] P.V. Hornbeck, et al., PhosphoSitePlus: a comprehensive resource for investigating the structure and function of experimentally determined post-translational modifications in man and mouse, *Nucl. Acids Res.* 40 (D1) (2012) D261–D270.
- [13] E.S. Yeh, M.A. Abt, E.G. Hill, Regulation of cell survival by HUNK mediates breast cancer resistance to HER2 inhibitors, *Breast Cancer Res. Treat.* 149 (1) (2015) 91–98.
- [14] E.V. Korobko, S.L. Kiselev, I.V. Korobko, Subcellular localization of MAK-V/Hunk protein kinase expressed in COS-1 cells, *Cell Biol. Int.* 28 (1) (2004) 49–56.
- [15] S.V. Kalinichenko, et al., Identification of Nedd4 E3 ubiquitin ligase as a binding partner and regulator of MAK-V protein kinase, *PLoS One* 7 (6) (2012) e39505.
- [16] B. Yang, S. Kumar, Nedd4 and Nedd4-2: closely related ubiquitin-protein ligases with distinct physiological functions, *Cell. Death. Differ.* 17 (1) (2010) 68–77.
- [17] L. Shearwin-Whyatt, et al., Regulation of functional diversity within the Nedd4 family by accessory and adaptor proteins, *Bioessays* 28 (6) (2006) 617–628.
- [18] M. Sudol, The WW module competes with the SH3 domain? *Trends Biochem. Sci.* 21 (5) (1996) 161–163.
- [19] S.V. Kalinichenko, P.N. Vikhreva, I.V. Korobko, Interaction between MAK-V protein kinase and synaptopodin, *Biochemistry (Mosc.)* 76 (2) (2011) 196–201.
- [20] P. Mundel, et al., Synaptopodin: an actin-associated protein in telencephalic dendrites and renal podocytes, *J. Cell Biol.* 139 (1) (1997) 193–204.
- [21] T. Deller, et al., Synaptopodin-deficient mice lack a spine apparatus and show deficits in synaptic plasticity, *Proc. Natl. Acad. Sci. U. S. A.* 100 (18) (2003) 10494–10499.
- [22] H. Stenmark, et al., Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion, *Cell* 83 (3) (1995) 423–432.
- [23] K. Komurov, et al., Comprehensive mapping of the human kinome to epidermal growth factor receptor signaling, *J. Biol. Chem.* 285 (27) (2010) 21134–21142.
- [24] E.S. Yeh, et al., Hunk is required for HER2/neu-induced mammary tumorigenesis, *J. Clin. Invest.* 121 (3) (2011) 866–879.
- [25] K. Phelps-Polirer, et al., Co-targeting of JNK and HUNK in resistant HER2-positive breast cancer, *PLoS One* 11 (4) (2016) e0153025.
- [26] D.G. Hardie, D. Carling, N. Halford, Roles of the Snf1/Rkin1/AMP-activated protein kinase family in the response to environmental and nutritional stress, *Semin. Cell Biol.* 5 (6) (1994) 409–416.
- [27] F. Lozy, V. Karantz, Autophagy and cancer cell metabolism, *Semin. Cell Dev. Biol.* 23 (4) (2012) 395–401.
- [28] P. Honscheid, K. Datta, M.H. Muders, Autophagy: detection, regulation and its role in cancer and therapy response, *Int. J. Radiat. Biol.* 90 (8) (2014) 628–635.
- [29] E.S. Yeh, et al., Hunk negatively regulates c-myc to promote Akt-mediated cell survival and mammary tumorigenesis induced by loss of Pten, *Proc. Natl. Acad. Sci. U. S. A.* 110 (15) (2013) 6103–6108.
- [30] K.R. Reed, et al., Hunk/Mak-v is a negative regulator of intestinal cell proliferation, *BMC Cancer* 15 (2015) 110.

- [31] H.P. Gardner, et al., Developmental role of the SNF1-related kinase Hunk in pregnancy-induced changes in the mammary gland, *Development* 127 (20) (2000) 4493–4509.
- [32] R.T. Fortner, et al., Premenopausal endogenous steroid hormones and breast cancer risk: results from the Nurses' Health Study II, *Breast Cancer Res.* 15 (2) (2013) R19.
- [33] B. Hoffman, D.A. Liebermann, Apoptotic signaling by c-MYC, *Oncogene* 27 (50) (2016) 6462–6472.
- [34] M. Quintela-Fandino, et al., HUNK suppresses metastasis of basal type breast cancers by disrupting the interaction between PP2A and cofilin-1, *Proc. Natl. Acad. Sci. U. S. A.* 107 (6) (2010) 2622–2627.
- [35] I.V. Korobko, et al., Proteinkinase MAK-V/Hunk as a possible diagnostic and prognostic marker of human breast carcinoma, *Arkh. Patol.* 66 (5) (2004) 6–9.
- [36] H.Y. Yang, et al., p27 Kip1 inhibits HER2/neu-mediated cell growth and tumorigenesis, *Oncogene* 20 (28) (2001) 3695–3702.
- [37] J. Zambrano, E.S. Yeh, Autophagy and apoptotic crosstalk: mechanism of therapeutic resistance in HER2-positive breast cancer, *Breast Cancer (Auckl)*. 10 (2016) 13–23.
- [38] Y. Avalos, et al., Tumor suppression and promotion by autophagy, *BioMed Res. Int.* 2014 (2014) 603980.
- [39] A. Vazquez-Martin, C. Oliveras-Ferreras, J.A. Menendez, Autophagy facilitates the development of breast cancer resistance to the anti-HER2 monoclonal antibody trastuzumab, *PLoS One* 4 (7) (2009) e6251.
- [40] K. Sharma, et al., Cytotoxic autophagy in cancer therapy, *Int. J. Mol. Sci.* 15 (6) (2014) 10034–10051.
- [41] S. Cufi, et al., The anti-malarial chloroquine overcomes primary resistance and restores sensitivity to trastuzumab in HER2-positive breast cancer, *Sci. Rep.* 3 (2013) 2469.
- [42] S. Chen, et al., Protective autophagy promotes the resistance of HER2-positive breast cancer cells to lapatinib, *Tumour Biol.* 37 (2) (2016) 2321–2331.
- [43] L.A. Gyorffy, A.C. Eklund, C. Denkert, J. Budczies, Q. Li, Z. Szallasi, An online survival analysis tool to rapidly assess the effect of 22,277 genes on breast cancer prognosis using microarray data of 1809 patients, *Breast Cancer Res. Treat.* 123 (3) (2010) 723–731.
- [44] A.M. Szasz, L.A. Nagy A, S. Forster, K. Hark, J.E. Green, A. Boussioutas, R. Busuttil, A. Szabo, B. Gyorffy, Cross-validation of survival associated biomarkers in gastric cancer using transcriptomic data of 1,065 patients, *Oncotarget* 7 (August 2 (31)) (2016) 49322–49333, <http://dx.doi.org/10.18632/oncotarget.10337>.
- [45] C. Law, Y. Chen, W. Shi, G.K. Smyth, Voom: precision weights unlock linear model analysis tools for RNA-seq read counts, *Genome Biol.* 15 (2014) R29.
- [46] G. Smythe, Linear models and empirical Bayes methods for assessing differential expression in microarray experiments, *Stat. Appl. Genet. Mol. Biol.* 3 (2004) 1.