JUNB promotes the survival of Flavopiridol treated human breast cancer cells ☆

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ABSTRACT

Chemotherapy resistance is a major obstacle to achieving durable progression-free-survival in breast cancer patients. Identifying resistance mechanisms is crucial to the development of effective breast cancer therapies. Immediate early genes (IEGs) function in the initial cellular reprogramming response to alterations in the extracellular environment and IEGs have been implicated in cancer cell development and progression. The purpose of this study was to investigate the influence of kinase inhibitors on IEG expression in breast cancer cells. The results demonstrated that Flavopiridol (FP), a CDK9 inhibitor, effectively reduced gene expression. FP treatment, however, consistently produced a delayed induction of JUNB gene expression in multiple breast cancer cell lines. Similar results were obtained with Sorafenib, a multi-kinase inhibitor and U0126, a MEK1 inhibitor. Functional studies revealed that JUNB plays a pro-survival role in kinase inhibitor treated breast cancer cells. These results demonstrate a unique induction of JUNB in response to kinase inhibitor therapies that may be among the earliest events in the progression to treatment resistance.

1. Introduction

Breast cancer is the most common cancer diagnosed in women in the United States [1]. Treatment of breast cancer patients has shifted in recent years from cytotoxic chemotherapy to therapies that target hormone and growth factor receptors and oncogenic signaling pathway components [2]. Targeted therapies have contributed to the improved survival rates of breast cancer patients in recent years, but resistance to targeted therapies is common and remains a significant problem in the treatment of breast cancer patients [2,3].

Cyclin dependent kinases (CDKs) are emerging as attractive targets for anti-cancer therapeutics due to their key roles in the control of cell cycle progression, cell survival and gene transcription [4,5]. Results from a recent Phase II clinical trial demonstrated a dramatic improvement in progression free survival of ER+ breast cancer patients treated with Palbocyclib, a CDK4/6 inhibitor [5]. As a component of the Positive Transcription Elongation Factor b complex CDK9 plays a key role in gene expression by phosphorylating the RNA Polymerase II (Pol II) C-terminal domain (CTD) on serine 2 (Pol IIser2p) and activating transcriptional elongation [6,7]. Flavopiridol (FP) is a semisynthetic alkaloid that inhibits CDK9 phosphorylation of Pol II, dramatically reducing Pol II mediated transcription [8,9]. FP was the first CDK inhibitor in clinical trials and FP has been extensively investigated in human lymphoma patients and more recently in pre-clinical and clinical studies in combination with trastuzumab and Sorafenib in breast cancer [8,10,11].

Immediate early genes (IEGs) are rapidly and transiently expressed in response to extracellular ligands and cell stressors [12]. Aberrant expression of IEGs, such as MYC, JUNB and FOS has been linked to a variety of human cancers, including breast cancer [13–15]. In this report we investigated the influence of FP treatment on CDK9 phosphorylation of Pol II and IEG expression in human breast cancer cell lines. The results indicated that FP treatment initially reduces the Pol IIser2p levels as well as the expression of MYC, JUNB and CEBPD. In addition, FP treatment

reduces the expression of MCL1, consistent with FP mediated programmed cell death [16]. We also demonstrate a unique delayed induction of JUNB gene expression in response to FP treatment that occurs in multiple human breast cancer cell lines. JUNB plays a well-established role as an oncogene in Hodgkin's lymphoma and anaplastic large cell lymphoma [17]. In breast cancer patients JUNB was identified among a subset of 27 genes up-regulated in primary inflammatory breast tumors [18]. In this report we demonstrate that JUNB plays a pro-survival role in breast cancer cells. The delayed induction of JUNB in response to FP treatment extends to other kinase inhibitors including Sorafenib and U0126, but does not include cytotoxic chemotherapy, i.e., doxorubicin. These results demonstrate a unique induction of JUNB in response to kinase inhibitors that may be among the earliest events in the progression to treatment resistance.

2. Materials and methods

2.1. Cell lines and reagents

The human breast cancer cell lines, SKBR3, MDA-MB-231, and MDA-MB-468 were purchased from ATCC and cultured in DMEM medium plus 10% FBS, 50 U/ml streptomycin, 50 U/ml penicillin, and 500 ng/ml Fungizone. MCF10CA1a (MCF10A^{met}) human breast cancer cells were a kind gift from Dr. Tsonwin Hai (Ohio State University) and were cultured in DMEM-F12 supplemented with 5% horse serum, 100 U/ml penicillin, 100 U/ml streptomycin and 500 ng/ml Fungizone. Cells were plated at ~60% confluence prior to treatment with the designated doses of Flavopiridol (FP), Actinomycin D, U0126 (Sigma); Doxorubicin and Sorafenib (Selleckchem).

2.2. RNA extraction and RT-PCR/Real Time PCR

Total RNA was isolated using the Roche High Pure RNA Isolation Kit. cDNA was synthesized using the Roche transcriptor first strand cDNA synthesis kit using both the oligo-dT and random hexamer primers. For Reverse Transcriptase PCR (RT-PCR) mRNA products were amplified (25–40 cycles) and the PCR products were electrophoresed using a 1% agarose gel containing 0.01% ethidium bromide and imaged using an AlphaImager (Alpha Innotech). For Real Time PCR, the reverse transcription products were amplified using the Roche LightCycler® 480 II Real-Time PCR System with SYBR green mastermix. PCR specificity was verified by assessing the melting curves of each amplification product. The fold change in specific mRNA levels was calculated using the comparative CT ($\Delta\Delta$CT) method [19]. The PCR primers (Sigma) are as follows: JUNB (forward: 5′-CACCAAGTGCCGGAAGCGGA-3′; reverse: 5′-AGGGGC AGGGGACGTTCAGA-3′), CEBPD (forward: 5′-AGAAGTTGGTGGA GCTGTCG-3′; reverse: 5′-GGTATGGGTCGTTGCTGAGT-3′), MYC (forward: 5′-AGAAGTTGGTGGAGCTGTCG-3′; reverse: 5′-GGTATGG GTCGTTGCTGAGT-3′), MCL1 (forward: 5′-AGAAGTTGGTGGAGCT GTCG-3′; reverse: 5′-GGTATGGGTCGTTGCTGAGT-3′), GAPDH (forward: 5′-AGAAGTTGGTGGAGCTGTCG-3′; reverse: 5′-GGTATGGGT CGTTGCTGAGT-3′), β-ACTIN (forward: 5′-GCTCGTCGTCGACAAC GGCTC-3′; reverse: 5′-CAAACATGATCTGGGTCATCTTCTC-3′).

2.3. Western blotting

Cell lysates were harvested using RIPA buffer containing protease, kinase, and phosphatase inhibitors. Protein lysates (30 μg) were resolved by SDS–PAGE and transferred to Immobilon-P PVDF membrane (Millipore). Membranes were blocked with 5% milk in Tris-buffered saline with 1% Tween-20 (TBST). Anti-JUNB (1:1000, Cell Signaling), anti-RNA Polymerase IISer2p (1:1000,

Abcam) anti-Cyclin D1 (1:5000, Cell Signaling), anti-Cleaved Caspase 3 (1:1000, Cell Signaling), anti-Tubulin (1:1000, Cell Signaling), anti-β-actin (1:2000, rabbit, Cell Signaling) antibodies were diluted in 5% milk-TBST overnight. After washing with TBST, membranes were probed with horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (1:10,000, Cell Signaling) (1 h). Membranes were developed using Pierce ECL 2 western blotting chemiluminescence substrate (Thermo Scientific).

2.4. siRNA based knockdown assay

JUNB siRNA interference was induced with short interfering RNA (siRNA) (Dharmacon) directed against the human JUNB gene. Controls received non-targeting siRNAs (NC siRNA) (Dharmacon). siRNAs were transfected into cells using the Amaxa Nucleofector II and Amaxa transfection kits (Lonza). Following siRNA nucleofection, cells were re-plated and cultured in complete media for 12 h followed by FP treatment.

2.5. Crystal violet staining

MCF10A^{met} breast cancer cells were grown to ~60% confluence and treated with JUNB or control siRNA, allowed to rest for 12 h, and treated with FP for 24 h. Cells were harvested, washed and stained with 0.5% crystal violet in 20% methanol.

2.6. Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of the fold changes for three independent experiments. Differences between samples were analyzed by *t*-tests using JMP. $P < 0.05$ was regarded to be statistically significant. Multiple treatment analyses were performed by one way ANOVA with Dunnett's test or the Student's *t* test where the fold change in mRNA levels was considered significant at $p < 0.05$.

3. Results

3.1. JUNB mRNA levels, exhibit a delayed induction in response to Flavopiridol (FP) treatment

To determine the influence of FP on IEG expression MCF10A^{met} cells were treated with FP and the expression of selected IEGs (JUNB, CEBPD and MYC) was assessed. FP treatment reduced JUNB, CEBPD and MYC mRNA levels (Fig. 1A). In addition, FP treatment reduced MCL1 mRNA levels (Fig. 1A). The initial decline in mRNA levels was maintained after 4 h of FP treatment for all genes except JUNB. After an initial decline, JUNB mRNA levels dramatically increased at 4 h post FP treatment (Fig. 1A). Co-treatment of MCF10A^{met} cells with FP + Actinomycin D blocked the delayed induction of JUNB mRNA levels, suggesting that the re-emergence of JUNB mRNA was mediated at the level of transcription (Fig. 1B). The delayed induction of JUNB mRNA levels is also reflected in JUNB protein levels in FP treated MCF10A^{met} cells (Fig. 1C). These results demonstrate that JUNB mRNA levels are uniquely induced in MCF10A^{met} cells despite the effective FP mediated reduction of Pol IISer2p levels.

3.2. JUNB mRNA levels increase despite FP mediated reduction in Pol IISer2p levels

FP is a highly selective CDK9 inhibitor [20]. To determine if FP treatment resulted in reduced levels of Pol IISer2p the levels Pol IISer2p were assessed in FP (300 nM) treated MCF10A^{met} cells. The results indicate that FP treatment reduces Pol IISer2p levels
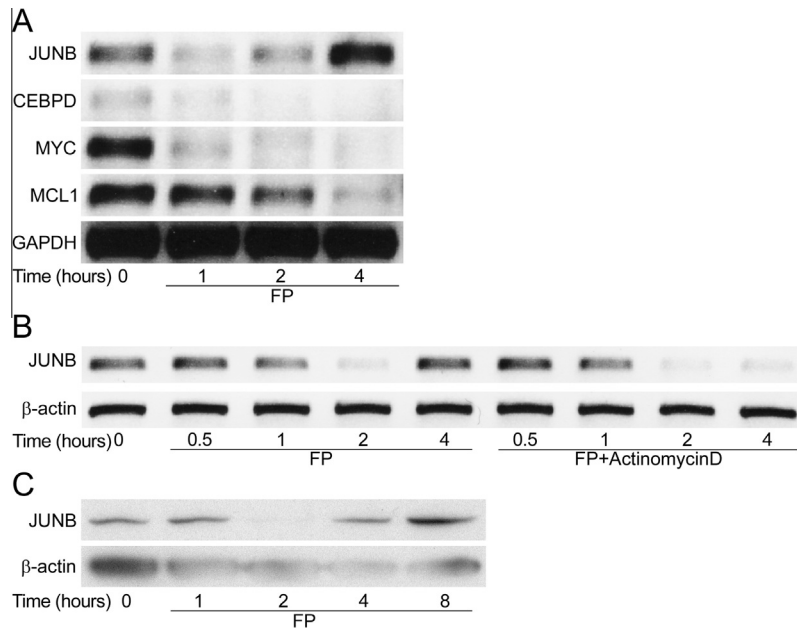
Fig. 1. JUNB is induced by Flavopiridol (FP) treatment. (A) MCF10A^met cells were treated with FP (300 nM) for 1, 2, or 4 h. JUNB, CEBPD, MYC and MCL1 mRNA levels were assessed by Real time PCR. GAPDH was used as the loading control. (B) MCF10A^met cells were treated with FP (300 nM) without or with Actinomycin D (10 μg/mL). JUNB mRNA levels were assessed at 0, 0.5, 1, 2 and 4 h by Real Time PCR. β-actin was used as the RNA loading control. (C) MCF10A^met cells were treated with FP (300 nM) for 0, 1, 2, 4 and 8 h. JUNB protein levels were assessed Western blot. β-actin was used as the loading control.

at every time point (2–24 h) (Fig. 2A). To determine if the effects of FP treatment on JUNB mRNA levels extended to additional human breast cancer cell lines JUNB mRNA levels were assessed in FP treated MCF10A^met, SKBR3, MDA-MB-231 and MDA-MB-468 human breast cancer cell lines. FP treatment induced an initial decline in JUNB mRNA levels which was followed by the delayed induction of JUNB mRNA levels in all five human breast cancer cell lines (Fig. 2B–E). The delayed induction of JUNB in response to FP treatment varied from ∼5 to 12-fold (Fig. 2B–E). MYC mRNA levels were not induced by FP treatment in any of the breast cancer cell lines tested demonstrating the relative specificity of the delayed induction response for JUNB (Fig. 2B–E).

3.3. JUNB plays a pro-survival role in FP-treated MCF10A^met cells

JUNB expression has been linked to the regulation of fundamental cell programs including cell cycle progression, genetic stability, programmed cell death and autophagy [21–24]. To assess the potential role of JUNB on MCF10A^met cell proliferation JUNB levels were reduced by siRNA knockdown and cyclin D_1, an indicator of cell proliferation was assessed. JUNB siRNA treatment had no effect on cyclin D1 levels, indicating that reducing JUNB levels did not alter cell proliferation (Fig. 3A). We next verified that FP treatment induced apoptosis by treating MCF10A^met cells with FP and assessing cleaved Caspase 3 levels. Cleaved caspase3 was detected as
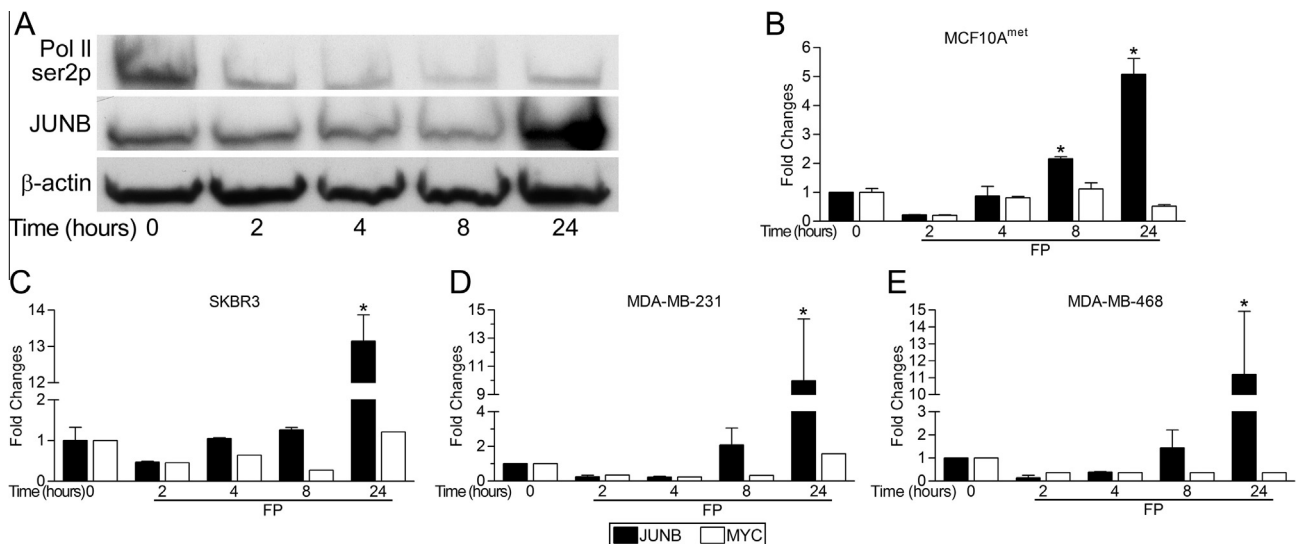

Fig. 2. JUNB is induced in multiple human breast cancer cell lines in response to FP treatment. (A) MCF10A^met cells were treated with FP (300 nM), a highly selective CDK9 inhibitor. Pol IIser2p and JUNB protein levels were assessed at 0–24 h by Western blot. β-actin was used as the loading control. (B–E) JUNB and MYC mRNA levels were assessed at 0–24 h after FP (300 nM) treatment by Real Time PCR. Data is presented as fold change in mRNA levels in FP treated cells compared to pretreatment (0 time) mRNA levels.
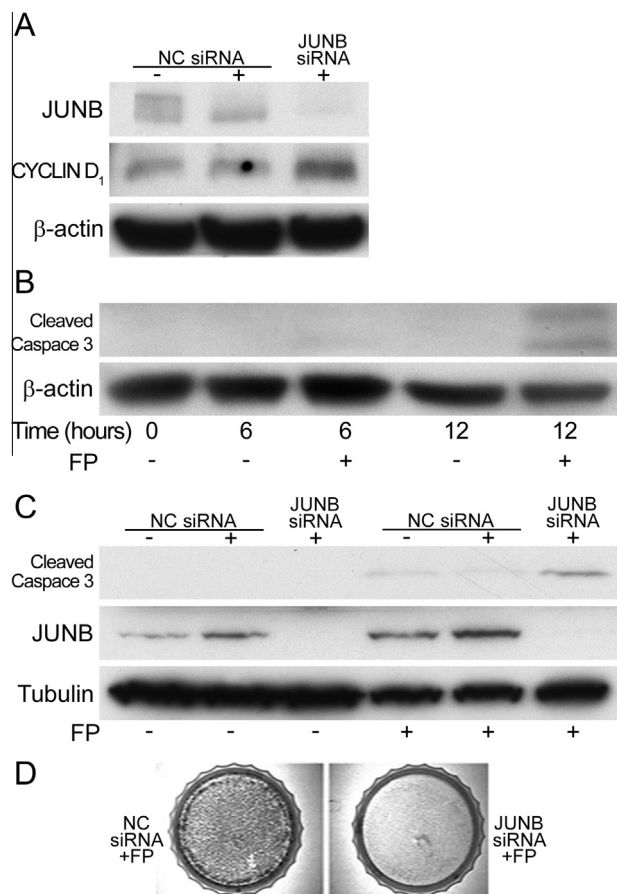
Fig. 3. JUNB plays a pro-survival role in FP treated MCF10A[met] cells. (A) MCF10A[met] cells were nucleofected with JUNB siRNA, Non-coding siRNA (NC-siRNA) or no siRNA and cultured in complete growth media for 24 h. JUNB and Cyclin D_1 protein levels were assessed by Western blot. β-actin was used as the loading control. (B) MCF10A[met] cells were treated with vehicle or FP (300 nM) for the designated times. Cleaved Caspase 3 protein levels were assessed by Western blot. β-actin was used as the loading control. (C) MCF10A[met] cells were nucleofected with NC-siRNA or JUNB siRNA, then treated with vehicle or FP (300 nM) for 12 h. Cleaved Caspase 3 and JUNB protein levels were assessed by Western blot. Tubulin was used as the loading control. (D). Cells were stained with crystal violet and photographed.

early as 6 h after FP treatment and cleaved Caspase3 was clearly visible after 12 h of FP treatment (Fig. 3B). To assess the role of JUNB in FP-induced apoptosis MCF10A[met] cells were treated with JUNB siRNA or noncoding siRNA and the induction of cleaved Caspase3 was assessed in response to FP (300 nM). The results demonstrated that treatment with JUNB siRNA effectively reduced JUNB protein levels but reducing JUNB expression alone did not induce Caspase3 cleavage (Fig. 3C). In contrast, reducing JUNB

protein levels was associated with a marked induction of cleaved Caspase3 in response to FP treatment compared to noncoding siRNA treated MCF10A[met] controls (Fig. 3C). We next assessed the survival of JUNB siRNA treated MCF10A[met] cells exposed to FP using crystal violet staining. The results documented the increase in FP mediated cell death in JUNB siRNA treated MCF10A[met] cells compared to controls (Fig. 3D). Collectively, these results demonstrate that JUNB plays a pro-survival role in FP treated MCF10A[met] cells.

3.4. JUNB is induced by kinase inhibitors (Sorafenib and U0126) but not cytotoxic chemotherapy (doxorubicin)

To determine if the FP-mediated induction of JUNB extended to therapeutic doses of small molecule inhibitors targeting kinases not directly associated with transcription we assessed JUNB expression in MCF10A[met] cells treated with Sorafenib (multi-kinase inhibitor) and U0126 (MEK1 inhibitor). Both Sorafenib (10 μM) and U0126 (50 μM) treatments induced an initial decline in JUNB mRNA levels, followed by an induction in JUNB mRNA levels after 24 h (Fig. 4A and B). To determine if the induction of JUNB mRNA levels is an obligatory component of the response of MCF10A[met] cells to chemotherapy treatments MCF10A[met] cells were treated with the anthracycline, doxorubicin. Doxorubicin is commonly used in the treatment of breast cancer [25]. The mechanism of action of doxorubicin involves DNA intercalation and inhibition of Topoisomerase 2 [26]. The results demonstrated that doxorubicin treatment (1 μM) does not induce JUNB (Fig. 4C). These results suggest that the delayed induction of JUNB is specific to small molecule kinase inhibitors and does not extend to doxorubicin.

4. Discussion

In this report we demonstrate the delayed induction of JUNB in response to FP, a CDK9 inhibitor. The unique induction of JUNB expression under conditions in which Pol IIser2p levels are reduced and gene expression levels are significantly diminished suggests that transcription complex components retain the capacity to associate with the JUNB proximal promoter in FP treated cells. Transcription of the JUNB gene is regulated by a "paused" Pol II control mechanism in which the Pol II transcription complex "pauses" ~50 nucleotides downstream from the transcription start site in a complex that includes two "pause factors", DSIF (DRB Sensitivity Inhibitory Factor) and NELF (Negative Elongation Factor) [27,28]. Recent studies indicate that HSP90 stabilizes NELF within the transcription complex in a gene specific manner [29]. This suggests that HSP90 could regulate the selective expression of JUNB by influencing NELF stability and pausing function [30]. In addition, as a member of the AP-I family of transcription factors,
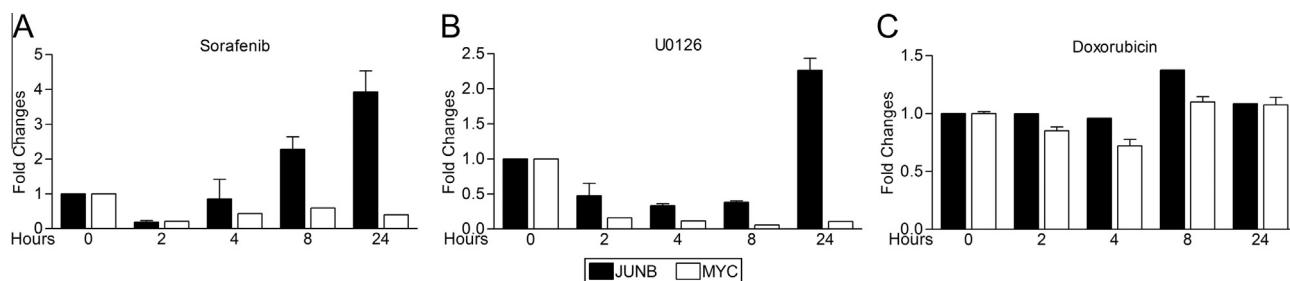

Fig. 4. JUNB gene expression is induced by treatment with Sorafenib and U0126, but not Doxorubicin. (A–C) MCF10A[met] cells were treated with Sorafenib (10 μM), U0126 (50 μM) or Doxorubicin (1 μM). JUNB and MYC mRNA levels were assessed at selected time points (0–24 h) after drug treatments by Real Time PCR. Data is presented as fold change in mRNA levels in drug treated cells compared to pretreatment (0 time) mRNA levels.

JUNB induces the expression of Cyclophilin40 (CYP40), an HSP90 co-chaperone protein [31]. Co-chaperones promote the interaction between chaperone proteins and their targets [30]. The delayed induction of JUNB that occurs at ~4 h after the initiation of FP treatment and continues for 24 h (last time point taken) could result from the delayed release of the HSP90 stabilized NELF/ "paused" Pol II complex. The kinase responsible for the phosphorylation events that result in the delayed induction of JUNB gene expression has not been identified but could include kinases capable of phosphorylating the Pol II CTD on serine 2, including Brd4 or CDK12 [6,32,33].

JUNB plays a key role in the pathogenesis of anaplastic large cell lymphoma but a functional role for JUNB in breast cancer has not been previously reported [34]. This study reveals a novel profile of JUNB induction that is delayed by several hours from the putative initiating event (small molecule kinase inhibitor treatment) and is also prolonged (~24 h). A delayed, "biphasic" induction of JUNB in response to cytokine and FP treatments has been previously reported but the biological significance of this novel JUNB expression pattern was not investigated [20,35]. Functional studies reported herein demonstrate that JUNB plays a pro-survival role in breast cancer cells in response to a lethal dose of FP. This is plausible as previous reports demonstrated that JUNB activates the transcription of survivin (BIRC5) an "inhibitor of apoptosis protein" that promotes cell survival [36]. In addition, JUNB protects pancreatic beta cells from cytotoxic fatty acid and cytokine treatments by inducing the expression of pro-survival target genes including Bcl-xl, Activating Transcription Factor3 (ATF3), CEBPD and X-box protein1 (XBP1) [37,38]. The results from this study are consistent with a role for JUNB mediated activation of an anti-apoptotic gene program that promotes cell survival in response to kinase inhibitor treatment.

In addition to inducing the expression of anti-apoptosis related genes, JUNB has also been associated with autophagy. Limited reports suggest that JUNB inhibits autophagy in response to non-physiological serum and growth factor starvation *in vitro* [24]. An extensive literature has accumulated in support of autophagy as a protective, pro-survival mechanism that promotes chemotherapy resistance [39]. Recent studies in chronic lymphocytic leukemia (CLL) cell lines and CLL patients demonstrate that FP induces a complex response that includes ER stress and a protective induction of autophagy that is associated with chemotherapy resistance [40]. In addition, direct evidence linking JUNB with chemotherapy resistance was reported in a global screening study in which JUNB was among a limited subset of 170 out of 15,906 human cDNAs tested for their ability to confer resistance to kinase inhibitor therapy [41]. JUNB transcriptional activity is activated by Jun N-terminal Kinase (JNK) phosphorylation, suggesting that combination therapy that includes the JNK inhibitor SP600125 may reduce the development of chemotherapy resistance [42,43]. In breast cancer patients, treatment resistance underlies disease recurrence, metastases development and a poor prognosis [44]. The results from this study demonstrate the novel induction of JUNB as an early event in the progression to treatment resistance.

Acknowledgments

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