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AhR ligands reactivate LINE-1 retrotransposon in triple-negative breast cancer cells MDA-MB-231 and non-tumorigenic mammary epithelial cells NMuMG

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Abstract

Breast cancer is the most common cancer type in females worldwide. Environmental exposure to pesticides affecting hormonal homeostasis does not necessarily induce DNA mutations but may influence gene expression by disturbances in epigenetic regulation. Expression of long interspersed nuclear element-1 (LINE-1) has been associated with tumorigenesis in several cancers. In nearly all somatic cells, LINE-1 is silenced by DNA methylation in the 5´UTR and reactivated during disease initiation and/or progression. Strong ligands of aryl hydrocarbon receptor (AhR) activate LINE-1 through the transforming growth factor-β1 (TGF-β1)/Smad pathway. Hexachlorobenzene (HCB) and chlorpyrifos (CPF), both weak AhR ligands, promote cell proliferation and migration in breast cancer cells, as well as tumor growth in rat models. In this context, our aim was to examine the effect of these pesticides on LINE-1 expression and ORF1p localization in the triple-negative breast cancer cell line MDA-MB-231 and the non-tumorigenic epithelial breast cell line NMuMG, and to evaluate the role of TGF-β1 and AhR pathways. Results show that 0.5 μM CPF and 0.005 μM HCB increased LINE-1 mRNA expression through Smad and AhR signaling in MDA-MB-231. In addition, the methylation of the first sites in 5´-UTR of LINE-1 was reduced by pesticide exposure, although the farther sites remained unaffected. Pesticides modulated ORF1p localization in MDA-MB-231: 0.005 μM HCB and 50 μM CPF increased nuclear translocation, while both induced cytoplasmic retention at 0.5 and 5 μM. Moreover, both stimulated double-strand breaks, enhancing H2AX phosphorylation, coincidentally with ORF1p nuclear localization. In NMuMG similar results were observed, since they heighten LINE-1 mRNA levels. CPF effect was through AhR and TGF-β1 signaling, whereas HCB action depends only of AhR. In addition, both pesticides increase ORF1p expression and nuclear localization. Our results provide experimental evidence that HCB and CPF exposure modify LINE-1 methylation levels and induce LINE-1 reactivation, suggesting that epigenetic mechanisms could contribute to pesticide-induced breast cancer progression.

Keywords

Hexachlorobenzene, chlorpyrifos, breast cancer, aryl hydrocarbon receptor, long interspersed nuclear element-1.

1.Introduction

Agriculture is one of the main economic activities in developing countries, so significant amounts of pesticides are found in the environment. Different studies have linked breast cancer risk with pesticide exposure, including organochlorine pesticide hexachlorobenzene (HCB) and organophosphate chlorpyrifos (CPF) [1, 2]. Although banned, HCB is released as a byproduct of chlorinated solvent manufacture [3] and has been detected in human samples such as breast adipose tissue, serum and milk [1, 4-7]. HCB promotes epithelial cell proliferation, preneoplastic lesions and alterations in mammary gland development as well as breast cancer cell migration and invasion, metastasis and angiogenesis [8]. Furthermore, HCB acts as an endocrine disruptor (ED) [9,10] and has been classified as a probable human carcinogen [11]. In turn, CPF is a current-use insecticide in fruit trees and soybeans and its presence has been documented in waters and soils [12-13]. Its presence was reported in plasma [14] as well as in colostrums and mature milk samples from rural mothers [15]. In addition, CPF metabolite (3,5,6-trichloro-2-pyridinol) has been found in human urine samples collected from eight countries [16]. CPF alters the endocrine balance and promotes hyperplasia in mammary gland [17], as well as increases mammary tumor incidence in rats [18]. Although these pesticides belong to different chemical families, both are associated with mammary carcinogenesis and weakly bind to the aryl hydrocarbon receptor (AhR) [19, 20]. AhR activation can trigger membrane actions, releasing c-Src from its cytosolic AhR complex which phosphorylates a variety of growth factor receptors [21], and nuclear actions, by which

AhR modulates the expression of genes involved in cell proliferation, differentiation, and/or apoptosis [22].

Breast cancer is the most frequently diagnosed disease and the main cause of cancer death among women [23]. Transforming growth factor-β1 (TGF-β1) is involved in mammary morphogenesis, as well as in the development and progression of breast cancer [24]. The AhR and TGF-β1 signaling pathways are interrelated and regulate several common processes including cell proliferation, differentiation, migration, invasion, and apoptosis [25, 26]. For instance, HCB exposure in breast cancer cells MDA-MB-231 and non-tumorigenic mammary epithelial cells NMuMG, promotes the phosphorylation of c-Src through AhR, leading to the activation of TGF-β1 signaling and an increase in cell migration and invasion [27, 28]. Environmental factors such as metals, persistent organic pollutants and EDs may modulate epigenetic changes [29], which are more frequent in tumor cells than genetic mutations. One of the modifications occurring in the first stages of malignant transformation is the wide hypomethylation of the genome, which affects repetitive transposable genetic elements such as the long interspersed nuclear element-1 (LINE-1) [30]. LINE-1 codes for two proteins: ORF1p, with nucleic acid binding activity, and ORF2p, an endonuclease and reverse transcriptase [31]. After transcription, mRNA is exported to the cytoplasm, where ORF1p and ORF2p are translated. In the cytoplasm, these proteins associate with their own mRNA to form ribonucleoproteins that will be imported into the nucleus. Then, ORF2p retrotranscribes the mRNA and nicks the genomic DNA, allowing cDNA to integrate into the genome [31]. This process is inhibited in somatic tissues by genetic and epigenetic mechanisms; however, aberrant expression of ORF1p and ORF2p and new somatic insertions have been detected in epithelial cancers [32, 33]. These insertions affect the genome by interrupting genes, generating DNA breaks, and altering the splicing and frequency of recombination, contributing to genomic instability [34]. LINE-1 retrotransposition create DNA double-strand breaks (DSBs) in breast cancer cells indicated by an accumulation of phosphorylated histone H2AX (γ-H2AX) foci [35], an early step in the cellular response to DSBs [36]. The demethylation of the LINE-1

internal promoter region is one of the mechanisms regulating LINE-1 transcription [37]. Studies have reported an association between the levels of ORF1p and ORF2p and the stage of mammary tumor development, as well as between the subcellular localization of these proteins and patient survival [38]. Moreover, benzopyrene, a strong ligand of AhR, induces LINE-1 reactivation and epithelial-mesenchymal transition in the human liver cancer cell line HepG2 through the TGF-β1 canonical pathway [39]. Based on these findings, we hypothesized that HCB and CPF may modulate LINE-1 expression through TGF-β1 signaling mediated by AhR in breast cancer cells MDA-MB-231 and non-tumorigenic mammary epithelial cells NMuMG. Therefore, the present study examined the effect of these pesticides on LINE-1 expression, methylation status and ORF1p localization.

2. Materials and methods

2.1 Chemicals

HCB (>99% purity, commercial grade) was obtained from Aldrich-Chemie GmbH & Co. (Steinheim, Germany). CPF (99% purity) was purchased from Chem. Service, Inc. (PA, USA). Anti-phospho-c-Src, anti-c-Src, anti-phospho-Histone H2A.X (Ser139), anti-Smad3 and anti-phospho-Smad3 antibodies were purchased from Cell Signaling Technology, Inc. (MA, USA). Anti-LINE-1 ORF1p antibody (MABC1152) was obtained from EMD Millipore Corporation (CA, USA). Anti-β-Actin and anti-GAPDH antibodies, dimethyl sulfoxide (DMSO), trypsin, glutamine, amiloride hydrochloride hydrate and inhibitors4,7-orthophenanthroline (PHE) and SB431542 were purchased from Sigma-Aldrich Chemical, Co. (MO, USA). Anti-AhR and anti-Histone 3 antibodies were purchased from Abcam, Ltd. (Cambridge, UK). The enhanced chemiluminescence kit (ECL) was obtained from GE Healthcare Life Sciences (Buckinghamshire, UK). RPMI-1640 culture medium was obtained from HyClone Laboratories, Inc. (UT, USA). Random primers were purchased from Biodynamics (Buenos

Aires, Argentina). Moloney murine leukemia virus reverse transcriptase (M-MLV RT), cofactors for reverse transcription, and HpaII, HinfI and EcoRI enzymes were obtained from Promega Corporation (WI, USA). The BstUI enzyme was from New England BioLab (MA, USA). The kit Hot Firepol EvaGreen qPCR Mix Plus (ROX) was purchased from Solis Biodyne (Tartu, Estonia), and the specific oligonucleotides were obtained from Thermo Fisher (Perth, UK). All other reagents used were of analytical grade.

2.2 Cell culture and treatment

The human breast cancer cell line MDA-MB-231 (American Type Culture Collection) represents a triple-negative phenotype (ERα, PR and HER-2 negative), with a great degree of malignancy. The NMuMG cell line (Sigma-Aldrich) was derived from normal mammary gland tissue of a NAMRU adult mouse. The cells were cultured at 37 °C in a 5% CO_2 incubator with RPMI-1640 (for MDA-MB-231) or MEM (for NMuMG) supplemented with10% fetal bovine serum (FBS), 1% antibiotic–antimycotic mixture (10,000 Units/ml penicillin, 10 mg/ml streptomycin sulfate, and 25 µg/ml amphotericin B), and 1% glutamine. After 24 h of starvation, the cells at 70–80% confluence were exposed to HCB or CPF dissolved in ethanol (EtOH). For dose-response assays, the cells were exposed for 15 min, 24 or 48 h to HCB (0.005, 0.05, 0.5, and 5 µM), CPF (0.05, 0.5, 5, and 50 µM) or vehicle in RPMI supplemented with 5% FBS. For time-course studies, the cells were treated with CPF (0.5 µM) or vehicle in RPMI supplemented with 5% FBS for 5, 15, and 30 m, as well as 2, 6, and 24 h. The final EtOH concentration in each treatment was 0.5% and had no influence on the parameters analyzed as shown previously [40]. When indicated, the cells were pretreated with 2 µM SB431542, which is an inhibitor of the TGF-β1 canonical pathway, or 5 µM PHE, which is an antagonist of AhR. Both inhibitors were dissolved in DMSO. Then, pesticides or vehicle were added to the media in the presence or absence of the inhibitors. All assays were performed at cell passages 6 to 15.

This research work was carried out using environmentally relevant doses of HCB and CPF. The highest HCB dose used (5 μM) was similar to that found in human serum from a rural population highly exposed to airborne HCB [41]. In addition, different studies have reported HCB concentration comparable to 0.05 μM in serum of mothers at the time of giving birth in China [6], and in umbilical cord serum in France [42]. Finally, other authors have reported HCB levels close to 0.005 μM in China [43] and Germany [44]. On the other hand, the lowest CPF concentration (0.05 μM) is similar to environmental values found in water or soil [13], while the 0.5 μM dose is comparable to CPF levels reported in water from Thailand [45]. In addition, higher doses were found in sediments [46]. Huen et al. [14] have reported CPF levels in plasma from womens and newborns living in an agricultural community which ranged from 0-1726 ng/mL (0-4.9 μM). Furthermore, CPF was detected in breast milk from nursing mothers in India which ranged from 8.5–355 μg/L (0.02-1 μM) [47].

2.3 Western blotting

After treatment, total cell protein lysates were prepared as previously described by Miret et al. [27]. For subcellular fractioning, the nuclear and cytosolic fractions were separated by differential centrifugation as previously reported [48]. The purity of each fraction was assessed by examination of nuclear and cytosolic-specific marker molecules Histone 3 and GAPDH, respectively. Protein concentration was determined as previously described [49], and 40 μg of protein was resolved by 10-12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were then blotted for phospho-Smad3 (1:250), phospho-c-Src (1:500), and then re blotted for Smad3 (1:500), c-Src (1:500), AhR (1:500), ORF1p (1:500), Histone 3 (1:500), GAPDH (1:2000) and β-Actin (1:1000) as previously reported [27].

2.4 RNA preparation and reverse transcription (RT)-quantitative PCR (qPCR)

Total RNA was isolated using TRI-reagent/chloroform extraction according to the manufacturer's protocol. Then, 1 mg of total RNA was reverse-transcribed using the M-MLV RT kit with random primers as previously described [27]. Expression levels of LINE-1 mRNA were analyzed using specific primers for the coding region of ORF1 (LINE-1 ORF1) and ORF2 (LINE-1 ORF2) (Table 1). The SYBR-Green I/Q Taq DNA polymerase mix was used on the 7500 Fast Real-time PCR System (Bio-Rad, CA, USA). Cycling conditions were as follows: denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 30 s (40 cycles). The specificity of the primer set was monitored by analyzing the dissociation curve, and the relative mRNA quantification was performed using the comparative ΔΔCt method with GAPDH as the housekeeping gene.

2.5 Bioinformatics

LINE-1 promoter regions were analyzed for CpG islands by using the Meth Primer software(http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). A CpG island was defined as a 200-bp DNA sequence with a calculated percentage of CpGs over 50% and a calculated vs expected CpG distribution over 0.60. These regions were also checked for restriction sites for BstUI, HinfI and HpaII to evaluate the number of methylation-sensitive sites. PCR primers were designed with Vector NTI Suite 6.0 software (Infomax Inc., MD, USA).

2.6 DNA methylation-sensitive analysis

The DNA methylation status of the LINE-1 promoter was analyzed using a combination of single digestions with methylation-sensitive restriction enzymes and subsequent qPCR analysis [50]. Genomic DNA was isolated by using TRI-reagent/chloroform extraction according to the manufacturer's protocol. The total concentration of DNA was quantified by A280 and stored at -20 °C until needed. Equal quantities (1μg) of total DNA were digested with 7.5 units of EcoRI (Promega, WI, USA) to reduce the size of the DNA fragments and then

purified with phenol/chloroform extraction and EtOH precipitation. Then, 0.5 µg of EcoRI-cleaved DNA was incubated with 10 units of BstUI (1 h at 60 °C), HinfI (3 h at 37 °C), or HpaII (3 h at 37 °C) and 1X enzyme buffer, following the manufacturer's instructions. The digestion products were purified with the phenol/chloroform method. The relative expression level of the different DNA regions was analyzed by qPCR. The primer sequences are shown in Table 1. After initial denaturation at 95 °C for 15 min, the reaction mixture was subjected to successive cycles of denaturation at 95 °C for 15 s, annealing at 54-60 °C for 15 s and extension at 72 °C for 15 s (40 cycles). The methylation-sensitive restriction enzymes BstUI, HinfI and HpaII are unable to cut at methylated sites, allowing amplification of the fragment. A region without HpaII restriction sites (IC-1) and another region without BstUI or HinfI restriction sites (IC-2) were used as the internal controls. The relative degree of methylation was determined by plotting Ct values against the log input (internal control), which yielded standard curves for the quantification of unknown samples [51].

Name	**Sense (5'-3')**	**Antisense (5'-3')**	**Product size**
HpaII (a)	GAGGAGCCAAGATGGCCGAA	AATCACCGTCTTCTGCGTCG	75 pb
HpaII (b)	GTGAGCGACGCAGAAGACGG	CCCACTGTCTGGCACTCCCT	82 pb
HpaII (c) and IC (2)	GTCGCACCTGGAAAATCGGG	CCGAGCCAGGTGTGGGATAT	101 pb
BstUI /HinfI(a) and IC (1)	GTGGGCGCAGGCCAGTGTGT	TCCAGGTGCGACCGTCACCC	133 pb
HinfI (b)	TTAAGAAACGGCGCACCACG	GCCGCCTTGCAGTTTGATCT	113 pb

LINE-1 ORF1	CAAGTTGGAAAACACTCTGCAG	GGAGTATCTTTGTGGCGTTCT	109 pb
LINE-1 ORF2	TCGACACATACACTCTCCCAAG	GCCACAATTTCAGAGCCTGTT	82 pb
Human β-Actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	234 pb
Mouse β2-Microglobulin	CAAGTATACTCACGCCACCCA	GCAGGCGTATGTATCAGTCTC	219 pb

Table 1. Primer sequences for qPCR analyses.

2.7 Immunofluorescence

After treatment, cells grown on cover glasses were fixed, permeabilized and blocked with blocking buffer (1% BSA and 1% Triton X-100 in phosphate-buffered saline, PBS) for 30 min at 37 °C and later incubated with an anti-ORF1p (1:20) mouse monoclonal or γ-H2AX (1:400) rabbit polyclonal antibodies in 0.2% BSA and 1% Triton X-100 in PBS for 24 h at room temperature in a humidified chamber. Finally, cells were incubated with the secondary antibodies Alexa 488 anti-mouse IgG (1:2000) or Alexa 488 anti-rabbit IgG (1: 200) for 1 h at room temperature in the dark. Hoechst was used for nuclei staining. Microscopic images were obtained using an Olympus BX50 F-3 fluorescence microscope (Olympus Optical Co., Ltd, Tokyo, Japan). Random fields were chosen by counting at least 1000 cells/treatment at 600X magnification.

2.8 Statistical analysis

Data were evaluated by one-way ANOVA followed by Dunnett's post hoc test to identify significant differences between controls and treatments. For assays with inhibitors, we used Tukey post-hoc test to identify differences between all groups. The results represent the mean ± SD of at least three independent experiments. DNA methylation-sensitive analysis was carried using Mann Whitney U test and results represent the mean ± SEM. Differences were considered significant when p values were <0.05.

3. Results

3.1 Pesticide exposure induces changes in LINE-1 mRNA expression

LINE-1 expression in tumor tissues is associated with several cancer characteristics, including progression, cancer risk, and poor prognosis [52]. To evaluate LINE-1 mRNA expression, RT-qPCR studies were conducted, analyzing LINE-1 ORF1 and ORF2 mRNA levels. First, we analyzed LINE-1 induction profiles in MDA-MB-231 cells exposed to CPF (0.05, 0.5, 5, and 50 μM), HCB (0.005, 0.05, 0.5, and 5 μM) or vehicle for 48 h. Results showed an increase in LINE-1 mRNA expression levels at all CPF doses assayed, with similar tendencies for both ORF1and ORF2 sequences (Fig. 1A). However, no changes were observed in LINE-1 mRNA levels upon 48 h HCB treatment (data not shown). After analyzing HCB action at different times, we repeated the dose response study for 24 h and found an increase in LINE-1 mRNA levels for both ORF1 and ORF2 at 0.005 μM HCB (Fig. 1B). Given that both LINE-1 specific sequences ORF1 and ORF2 showed similar results, the following assays were conducted only on the LINE-1 ORF1 sequence.

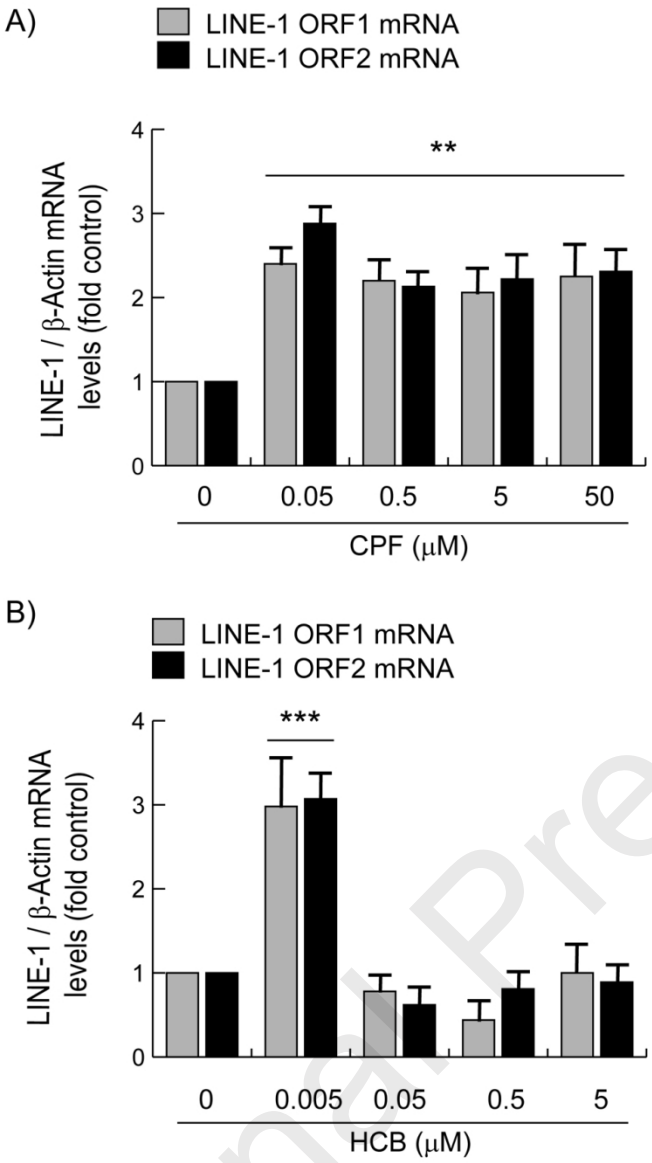

Fig. 1. HCB and CPF action on LINE-1 mRNA expression levels in MDA-MB-231 cells. LINE-1 ORF1 and ORF2 mRNA levels were evaluated by RT-qPCR. Cells were exposed to (A) CPF (0.05, 0.5, 5, and 50 μM) for 48 h or (B) HCB (0.005, 0.05, 0.5, and 5 μM) for 24 h. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (**p<0.01 and ***p<0.001; ANOVA and Dunnett's post hoc test).

3.2 CPF and HCB reduce the methylation status of LINE-1

LINE-1 retrotransposon expression is regulated by methylation of its internal promoter [53]. Considering the changes induced by pesticide exposure in the expression of LINE-1 mRNA, we next determined the methylation status of the 5´-UTR of the human LINE-1 sequence. Cells were treated with CPF (0.5 μM) for 48 h or HCB (0.005 μM) for 24 h, the doses and times which induced an increase in LINE-1 mRNA expression and which may also simulate the exposure concentrations of the general population [6, 16]. To search for potential sites of DNA methylation, the 5´-UTR was analyzed for CpG islands and restriction sites for HpaII, BstUI or HinfI methylation-sensitive restriction enzymes. One CpG island and 6 restriction sites were identified in the sequence studied (Fig. 2A). We observed that methylation was reduced by pesticide exposure at the first sites in the 5´-UTR (87, 152 and 216) but remained unaltered at the farther sites (283, 355 and 422). Specifically, the methylation status was decreased by CPF (0.5 μM) at sites 87 and 152, and by HCB (0.005 μM) at sites 87, 152 and 216 (Fig. 2B-G).

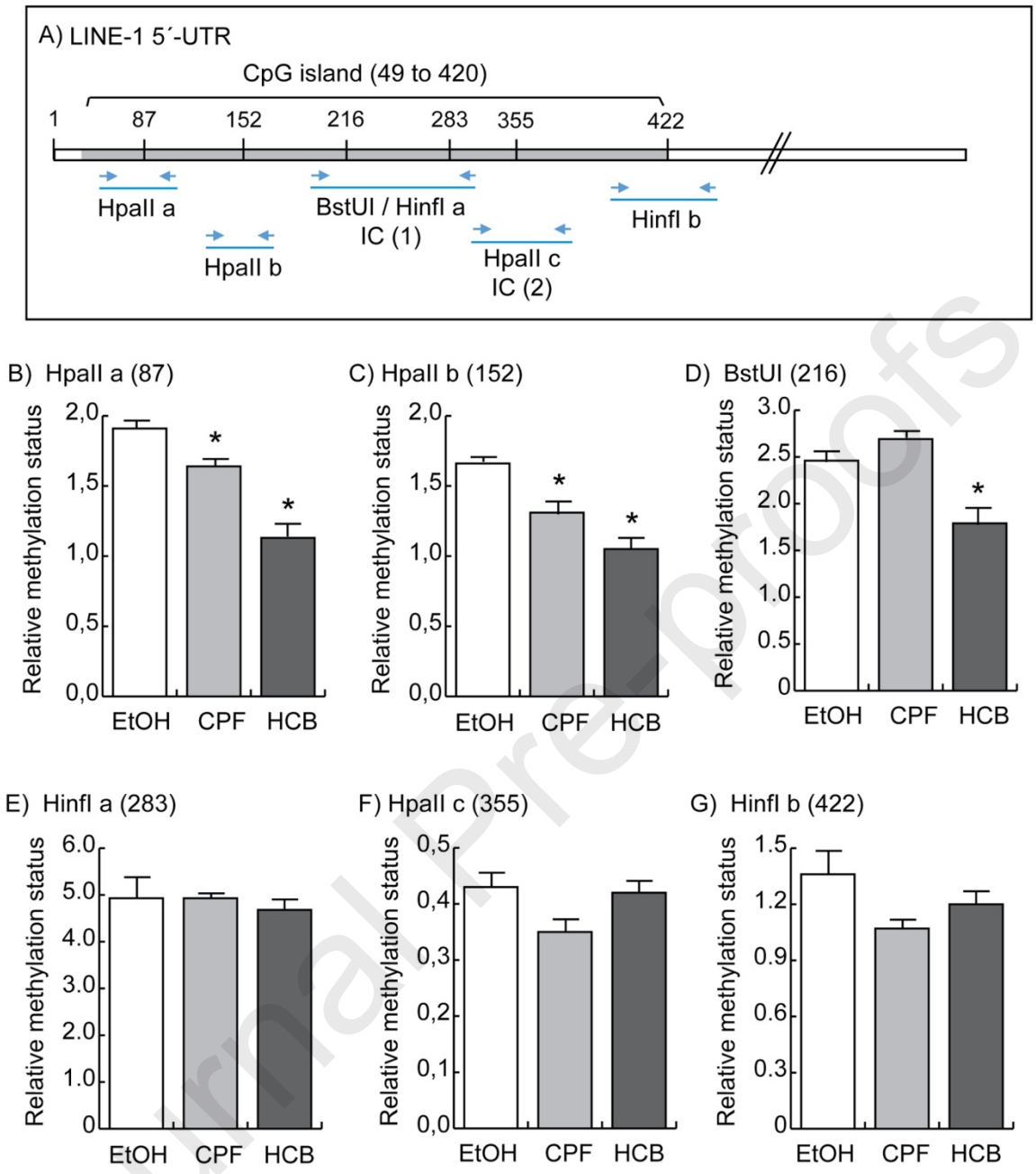

Fig. 2. Pesticide effects on LINE-1 methylation status in MDA-MB-231 cells. (A) Map of LINE-1 5´-UTR and its CpG island. Target sites for digestion by HpaII, BstUI and HinfI methylation-sensitive restriction enzymes are shown. Positions of PCR primers and their amplification products are indicated by arrows and lines, respectively. IC: internal control region. We named 1 to the first nucleotide from 5´UTR. (B-G) Cells were exposed to 0.5 µM CPF for 48 h, 0.005 µM HCB for 24 h or vehicle. Graphs show the relative methylation status of (B) HpaII a site (87), (C) HpaII b site (152), (D) BstUI site (216), (E) HinfI a site (283), (F) HpaII c site (355), and (G)HinfI b site

(422). Values are expressed as the mean ± SEM of at least three independent experiments (*p< 0.05, Mann Whitney U test).

3.3 CPF activates AhR and TGF-β1 signaling pathways in MDA-MB-231cells

A potential mechanism explaining the regulation of LINE-1 expression may be linked to the canonical TGF-β1 pathway, as reported for the strong ligand of AhR benzopyrene [39]. In this regard, we have previously observed that HCB promotes the AhR/c-Src axis, which in turn stimulates the canonical TGF-β1 pathway in the MDA-MB-231 cell line [27]. Therefore, we evaluated the action of CPF on AhR/c-Src and TGF-β1 signaling.

First, assays on AhR protein levels revealed an increase after 24 h of CPF (0.5, 5 and 50 μM) treatment (Fig. 3A). Then, analyses of c-Src phosphorylation levels in a time-course assay showed that 0.5 μM CPF increased phosphorylation at 5 and 15 min (Fig. 3B), in an AhR-dependent manner (Fig. 3C). Next, assays on the effect of CPF (0.05, 0.5, 5 and 50 μM) on Smad2 and 3 activation at 15 min showed an increase in the phosphorylation of both proteins at all doses assayed (Fig. 3D). Finally, a time-course study demonstrated that 0.5 μM CPF induced Smad2 phosphorylation only at 15 min, whereas it activated Smad3 at 15 min and 6 h (Fig. 3E).

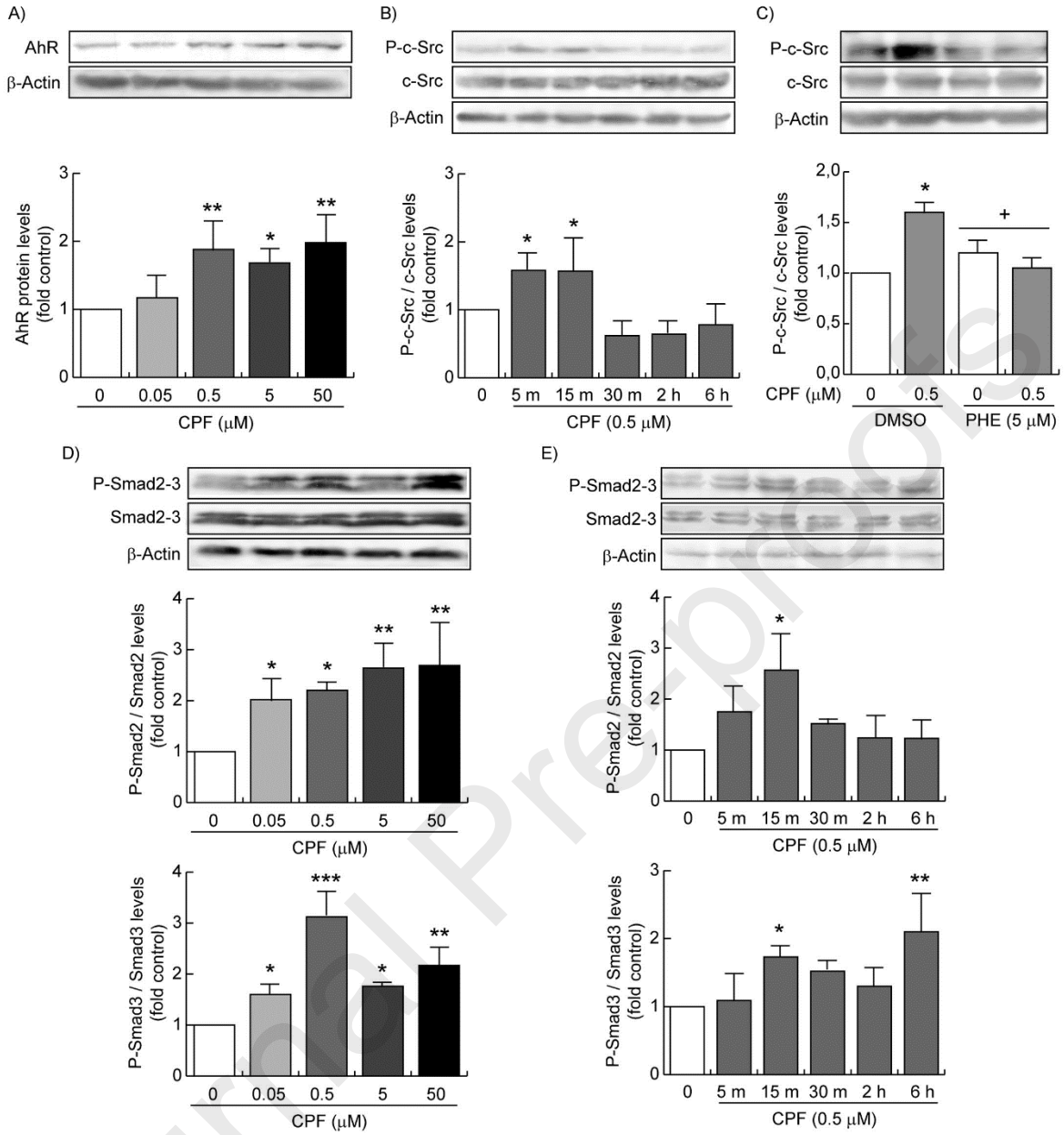

Fig. 3. CPF action on AhR and TGF-β1 signaling pathways in MDA-MB-231 cells. (A) AhR, (B-C) phospho (P)- and total-c-Src, and (D-E) P- and total-Smad2 and 3 protein levels. (A, D) Dose-response studies: cells were exposed to CPF (0.05, 0.5, 5, and 50 μM CPF) or vehicle for (A) 24 h or (C) 15 min. (B, E) Time-course studies: cells were treated with CPF (0.5 μM) or vehicle for 5, 15 and 30 min, 2 and 6 h. (C) Cells were pretreated with PHE (5 μM) or vehicle (DMSO) for 1 h and then treated with CPF (0.5 μM) in the presence or absence of inhibitor for 15 min. Whole-cell lysates were used to analyze protein levels by Western blot. The AhR protein/β-Actin protein ratio or phosphorylated protein/total protein ratio were normalized to control values. A representative Western blot from at least three independent experiments is shown in the upper panels (Smad2 top band, Smad3 lower band). Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed

as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p < 0.05, **p < 0.01 and ***p < 0.001; ANOVA and Dunnett's post-hoc test).

3.4 AhR and TGF-β1 signaling pathways regulate LINE-1 expression

Previously, we have observed that HCB exposure heightens cell migration and invasion through AhR and TGF-β1 signaling in MDA-MB-231 cells [27]. Based on those findings and studies reported by Reyes-Reyes et al. [39], who showed that benzopyrene induces LINE-1 reactivation through TGF-β1 and AhR pathways, we examined whether pesticide treatment could alter LINE-1 expression through these signaling in MDA-MB-231 cells.

In order to evaluate AhR participation in pesticide-induced LINE-1 mRNA expression, cells were pretreated for 1 h with AhR inhibitor PHE (5 μM) and then exposed to CPF (0.5 μM), HCB (0.005 μM) or vehicle during 48 or 24 h. Results clearly show that the presence of the inhibitor blocked the increase in LINE-1 mRNA levels exerted by CPF and HCB, unveiling an AhR-dependent mechanism (Fig. 4). In addition, and considering that pesticide-induced AhR activation promotes TGF-β1 signaling, we next examined TGF-β1 involvement in LINE-1 mRNA expression by pretreating MDA-MB-231 cells with TGF-β1 inhibitor SB431542 (2 μM) and then exposing them to CPF (0.5 μM), HCB (0.005 μM) or vehicle. Results again showed a blockade of CPF or HCB-induced LINE-1 expression upon inhibitor pretreatment, which reveals a TGF-β1-dependent mechanism (Fig. 4).

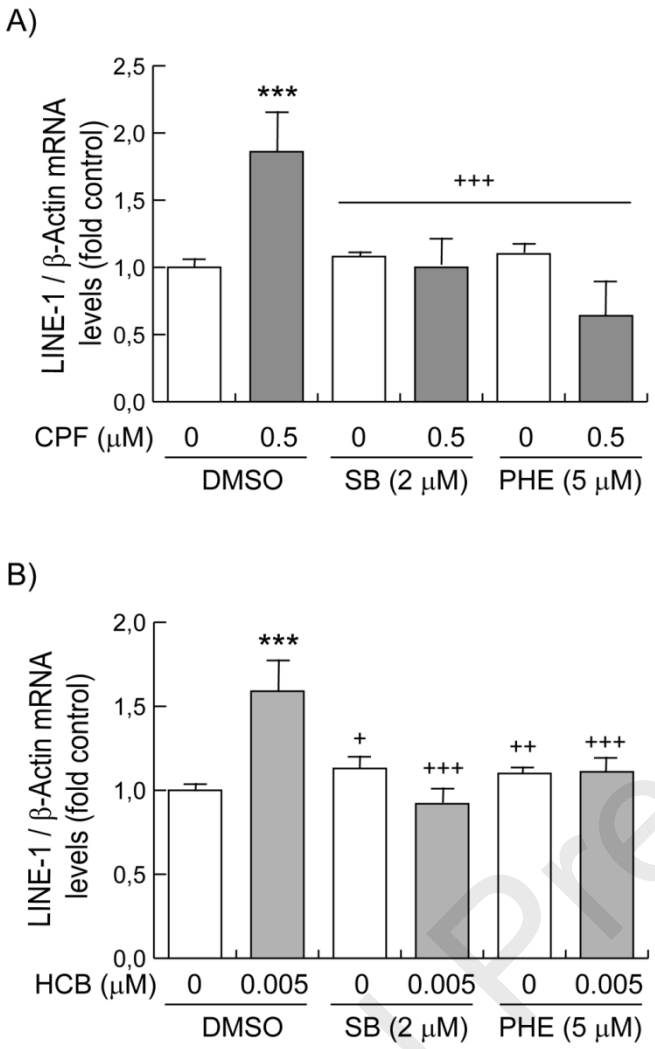

Fig. 4: Pesticide-induced LINE-1 expression depends on AhR and TGF-β1 signaling. Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle (DMSO) for 1 h and then treated with (A) CPF (0.5 μM) for 48 h or (B) HCB (0.005 μM) for 24 h, in the presence or absence of the inhibitors. LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (***p<0.001) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

3.5 CPF and HCB modulate ORF1p subcellular localization

Although LINE-1 encodes two proteins, ORF1p and ORF2p, in breast and hepatocellular carcinomas ORF1p shows higher expression levels than ORF2p and is likely to play important roles [54]. ORF1p is critical for LINE-1 retrotransposition, participating in ORF2p expression regulation, ribonucleoprotein complex formation and delivery to the chromosomal DNA, and/or assisting strand exchanges during retrotransposition [55]. For these reasons, we examined whether HCB or CPF exposure may affect ORF1p expression levels. In addition, the ORF1p subcellular localization was evaluated to assess LINE-1 retrotransposition activity.

MDA-MB-231 cells were treated with CPF (0.05, 0.5, 5, and 50 μM) for 48 h or HCB (0.005, 0.05, 0.5, and 5 μM) for 24 h, and ORF1p levels were evaluated by Western blot. Results revealed a reduction in ORF1p levels at 50 μM CPF (Fig. 5A); nevertheless, no changes were observed in this protein upon cell exposure to HCB (Fig. 5B). To determine whether pesticide exposure can induce ORF1p cytoplasm to nucleus translocation, ORF1p protein levels were examined in cytoplasmic and nuclear fractions, both of which revealed ORF1p expression (Fig. 5C-D). CPF increased nuclear translocation at 50 μM but promoted cytoplasmic retention at 0.5 and 5 μM (Fig. 5C). Besides, HCB increased nuclear translocation at 0.005 μM but induced cytoplasmic retention at higher doses (0.5 and 5 μM) (Fig. 5D). In addition, these results were verified by immunofluorescence assays, which further corroborated ORF1p nuclear localization at a low HCB dose (0.005 μM) and a high CPF dose (50 μM) (Fig. 6A, C).

Next, we have evaluated whether the pesticide action on ORF1p nuclear localization is dependent of AhR and TGF-β1/Smad pathways. For this purpose, MDA-MB-231 cells were pretreated with specific inhibitors (5 μM PHE for AhR and 2 μM SB431542 for TGF-β1 receptor I) for 1 h and then exposed to CPF (50 μM) for 48 h or HCB (0.005 μM) for 24 h, in the presence of inhibitors. ORF1p protein levels were examined in the nuclear fraction by Western blot, showing that CPF action is mediated by TGF-β1/Smad pathway, however when AhR signaling

was analyzed, a non-significative tendency was observed (Fig. 5E). In addition, HCB clearly induced ORF1p nuclear import through AhR and TGF-β1/Smad signaling (Fig. 5F).

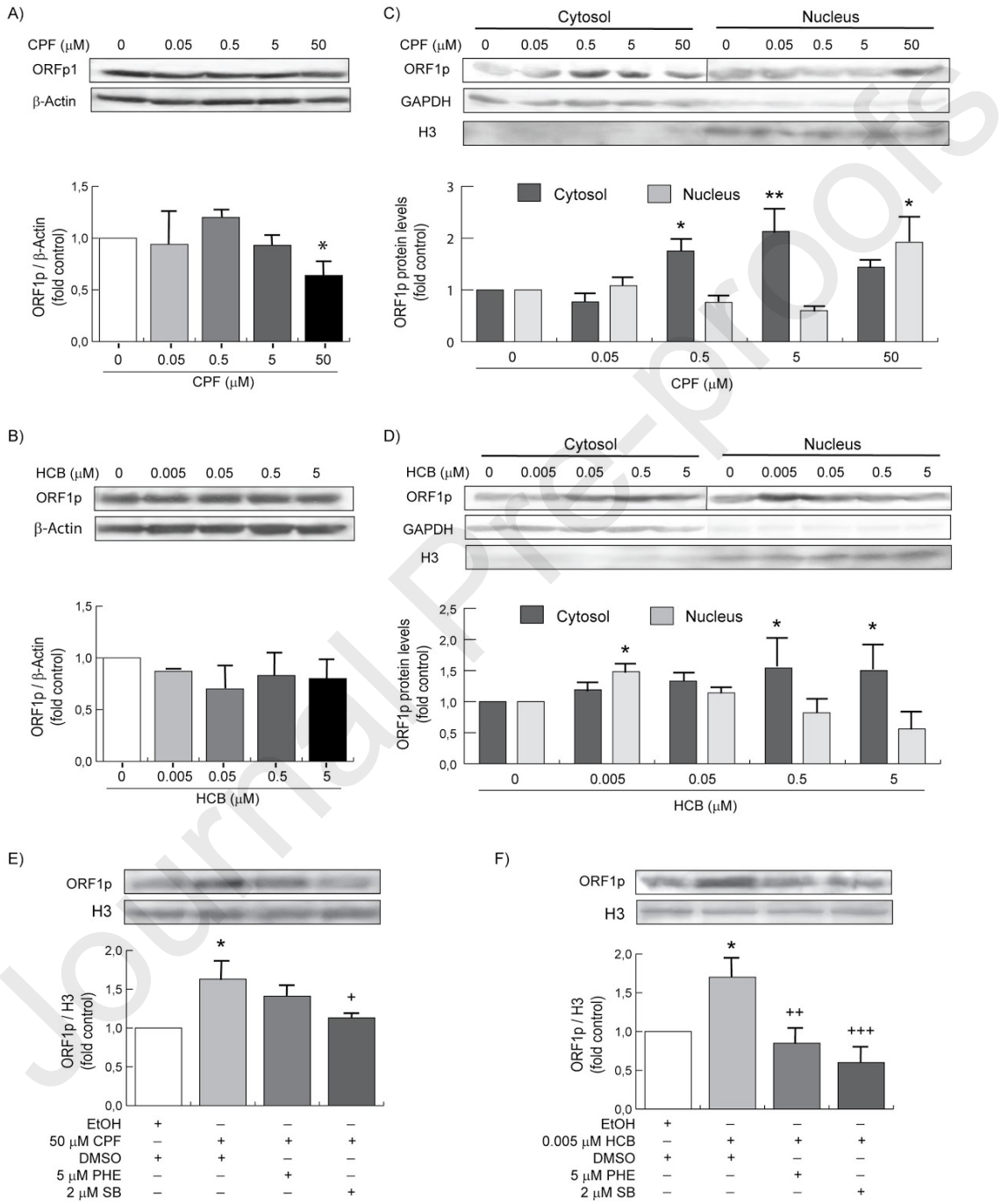

Fig. 5. Pesticide effects on ORF1p protein expression and localization in MDA-MB-231 cells. (A-B) ORF1p expression and (C-D) ORF1p cytoplasmic and nuclear localization and (E-F) ORF1p nuclear translocation analyzed by Western blot. Cells were exposed to (A, C) CPF (0.05, 0.5, 5, and 50 μM) or EtOH for 48 h, and (B, D) HCB (0.005, 0.05, 0.5 and 5 μM) or EtOH for 24 h. (E-F) Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle (DMSO) for 1 h and then exposed to (E) CPF (50 μM) for 48 h or (F) HCB (0.005 μM) for 24 h, in the presence of inhibitors. To normalize values, we used (A-B) anti-β-Actin antibody for whole cell lysates, (C-F) anti-Histone 3 (H3) for nuclei and (C-D) anti-GAPDH for cytosol. A representative Western blot is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p< 0.05 and **p< 0.01; ANOVA and Dunnett's post-hoc test) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

3.6 Pesticides induce DNA DSBs and H2AX phosphorylation

We observed that the lowest dose of HCB (0.005 μM) and the highest dose of CPF (50 μM) trigger ORF1p nuclear translocation in MDA-MB-231 cells (Fig. 6A, C). Given that aberrant expression of ORF1p and new somatic insertions produce genomic instability [34], then we evaluate if pesticides exposure may generate DNA DSBs. The phosphorylation of H2AX (γ-H2AX) is an early response to DSBs and can be observed flanking the DSB [56]. Therefore, we analyze the accumulation of γ-H2AX by immunofluoresce, finding that HCB and CPF exposure enhance γ-H2AX foci, coincidentally at the same doses that pesticides promote ORF1p nuclear localization. However, γ-H2AX remained unaltered at the pesticides doses where ORF1p is retained in the cytoplasm (Fig. 6B, D).

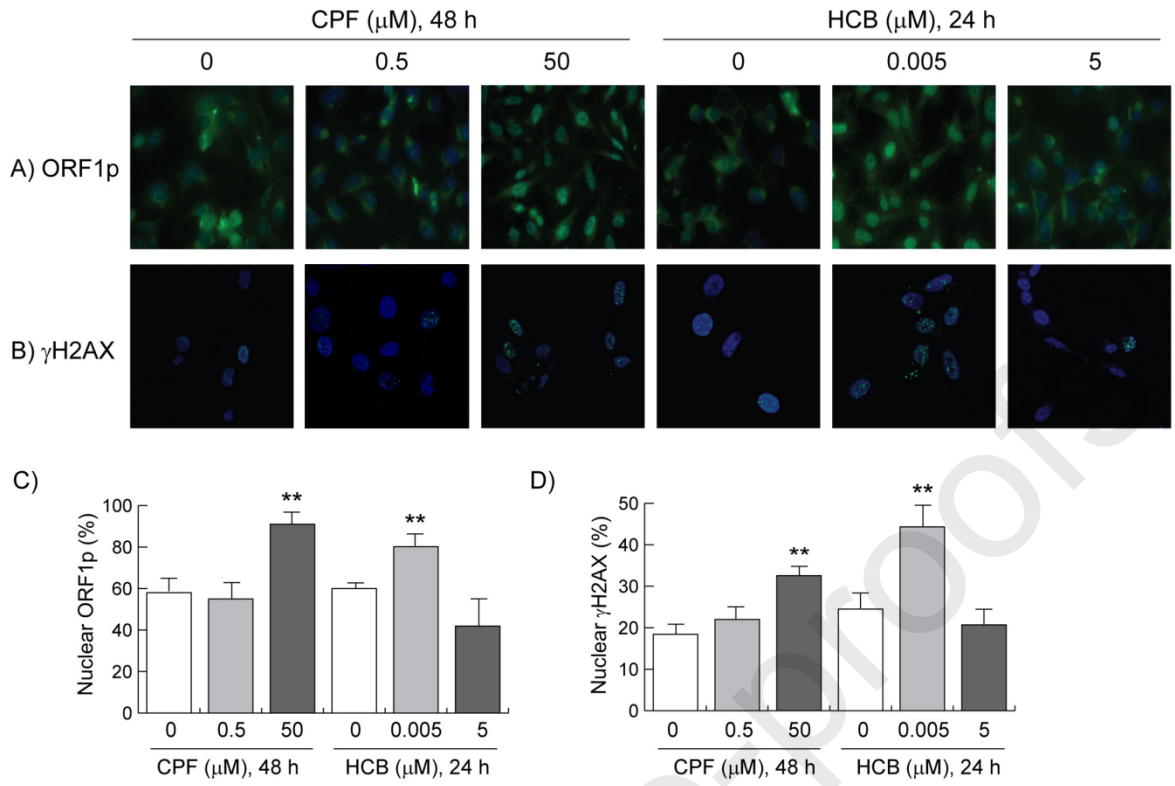

Fig. 6. ORF1p protein localization and H2AX phosphorylation. (A) Merge images of ORF1p or (B) γ-H2AX and Hoechst nuclear stain. Cells were exposed to HCB (0.005 and 5 μM), CPF (0.5 and 50 μM) or vehicle and protein expression was evaluated by immunofluorescence, by staining with specific antibodies. Magnification x 600. Graphics show the percentage of (C) ORF1p and (D) γ-H2AX-nuclear positive cells. We chose random fields counting at least 1000 cells/treatment. Data are expressed as means ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (**p< 0.01). ANOVA and Dunnett's post-hoc test.

3.7 Pesticide action on LINE-1 mRNA expression, and role of AhR and TGF-β1 signaling in non-tumorigenic mammary cells

As LINE-1 reactivation has been implicated in tumorigenesis [57], it was interesting to evaluate whether LINE-1 activation status could be modulated by pesticide exposure in epithelial mammary cells. For this reason, LINE-1 mRNA expression was evaluated in the non-

tumorigenic mammary epithelial cells NMuMG. Compared to MDA-MB-231 cells, NMuMG express significantly lower levels of LINE-1 mRNA and ORF1p (data not shown). NMuMG cells were exposed to CPF (0.05, 0.5, 5 and 50 μM), HCB (0.005, 0.05, 0.5 and 5 μM) or vehicle for 48 h and LINE-1 mRNA levels were evaluated by RT-qPCR. Data indicated clearly that both pesticides increases LINE-1 mRNA expression, CPF at 0.05, 0.5 and 5 μM (Fig. 7A) and HCB at 0.005 μM (Fig. 7B), with greater effect in the presence of CPF.

Previous results have shown that 0.05 μM HCB activates the AhR/c-Src/Smad3 axis in NMuMG cells, while AhR/c-Src is stimulated at 0.005 μM HCB [28, 58]. Herein, we studied CPF action on these signaling pathways. Cells were treated with CPF (0.05, 0.5, 5 and 50 μM) for 15 min and the phosphorylation levels of c-Src and Smad3 were analyzed by Western blot. As shown in Fig. 7C, c-Src was activated at all assayed doses but Smad3 phosphorylation was increased only at 5 and 50 μM CPF.

In order to evaluate if AhR and TGF-β1 pathways could be mediating pesticide-enhanced LINE-1 mRNA levels, NMuMG cells were pretreated for 1 h with 5 μM PHE (for AhR), or 2 μM SB431542 (for type I TGF-β receptor), and then exposed with pesticides for 48 h in the presence of inhibitors. We used 5 μM CPF, since it induced an enhancement in LINE-1 mRNA levels, as well as c-Src and Smad3 phosphorylation. RT-qPCR data showed that CPF-induced LINE-1 mRNA levels were prevented by both inhibitors, indicating that this action is AhR and TGF-β1-dependent (Fig. 7D). On the other hand, in regard to HCB exposure, herein we found that LINE-1 mRNA expression was stimulated at 0.005 μM. However, only c-Src phosphorylation was increased at this dose, without changes in Smad3 activation [58], therefore, we studied the role of AhR. We observed that PHE blocked the HCB-induced LINE-1 mRNA levels (Fig. 7E), showing that this action is mediated by AhR.

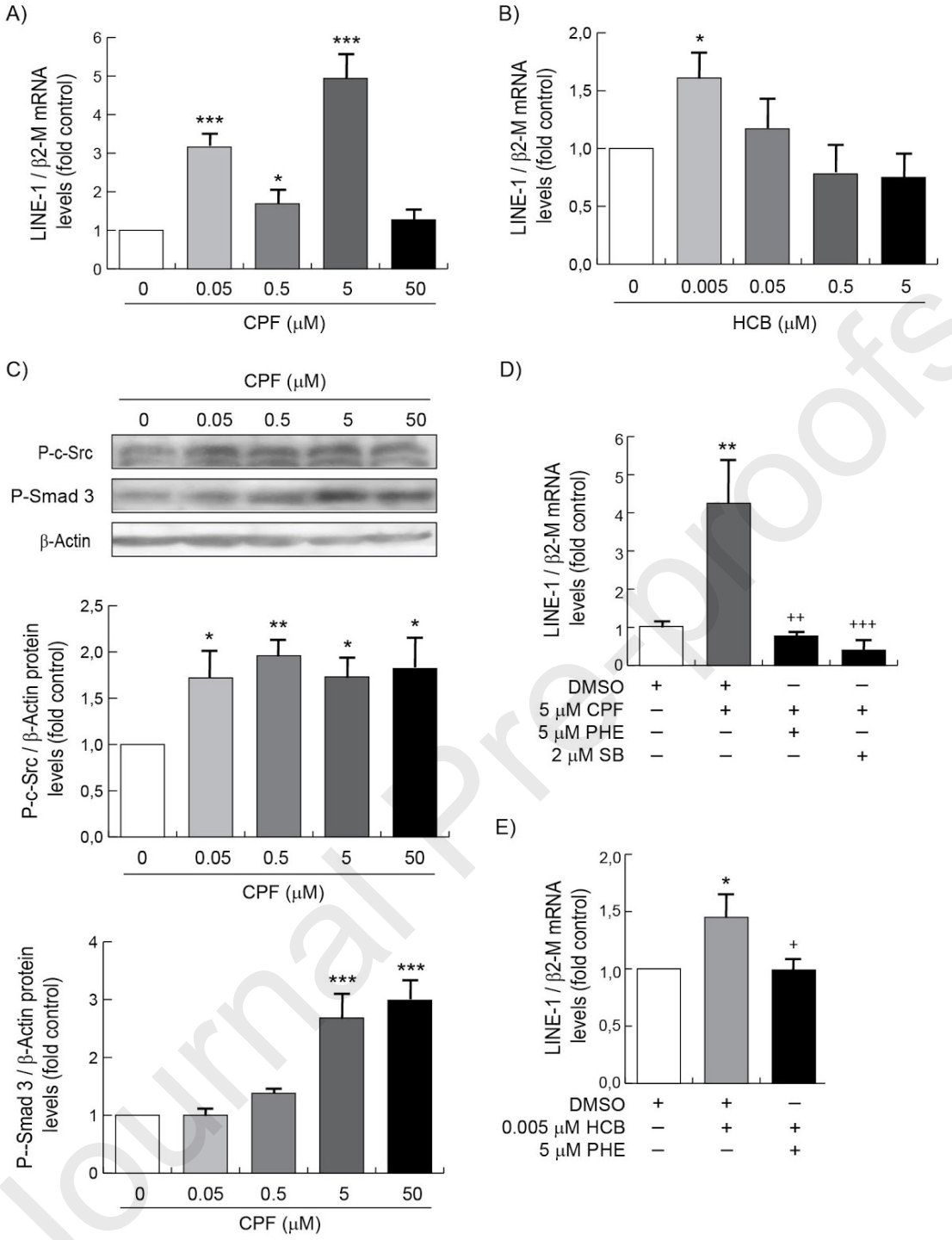

Figure 7: HCB and CPF modulate LINE-1 mRNA expression in non-tumorigenic mammary epithelial cells NMuMG. Role of AhR and TGF-β1 signaling. Cells were exposed to (A) CPF (0.05, 0.5, 5, and 50 μM) or (B) HCB (0.005, 0.05, 0.5, and 5 μM) for 48 h and LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. β2-microglobulin (β2-M) expression was used as a control to normalize the data. (C) Phospho (P)- c-Src, and P-Smad3 protein levels were determined by Western blot after 15 min of CPF treatment (0.05, 0.5, 5, and 50 μM). Values were normalized by immunoblotting using anti-β-Actin antibody. A Western blot from one representative

experiment is shown in the upper panel. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. (D-E) Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle (DMSO) for 1 h and then exposed to (D) CPF (5 μM) or (E) HCB (0.005 μM) for 48 h, in the presence of the inhibitor. LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (*p<0.05, **p<0.01, ***p<0.001; ANOVA and Dunnett's post-hoc test) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

3.8 CPF and HCB effects on ORF1p expression and localization in NMuMG cells

ORF1p nuclear expression compared to cytoplasmic expression is associated with poor patient survival [59]. Thus, ORF1p may be used as biomarker for cancer progression, and translocation of LINE-1 protein into the nucleus may serve as a risk indicator of poor prognosis. To evaluate if HCB and CPF exposure promote changes in ORF1p levels, NMuMG cells were treated for 48 h with CPF (0.05, 0.5, 5 and 50 μM) or HCB (0.005, 0.05, 0.5 and 5 μM), and analyzed by Western blot. Our data showed that 5 and 50 μM CPF, and 0.05, 0.5 and 5 μM HCB increase ORF1p expression levels in non-tumorigenic mammary epithelial cells NMuMG (Fig. 8A-B).

On the other hand, we examined ORF1p levels in cytosol and nucleus, with the aim of investigating whether pesticide treatment alters the localization of this protein. Western blot analysis show that 0.05-5 μM CPF induced ORF1p nuclear import (Fig. 8C). In addition, HCB enhanced both, nuclear and cytosolic ORF1p levels at the same doses as the increase in the total protein was observed (0.05-5 μM) (Fig. 8D).

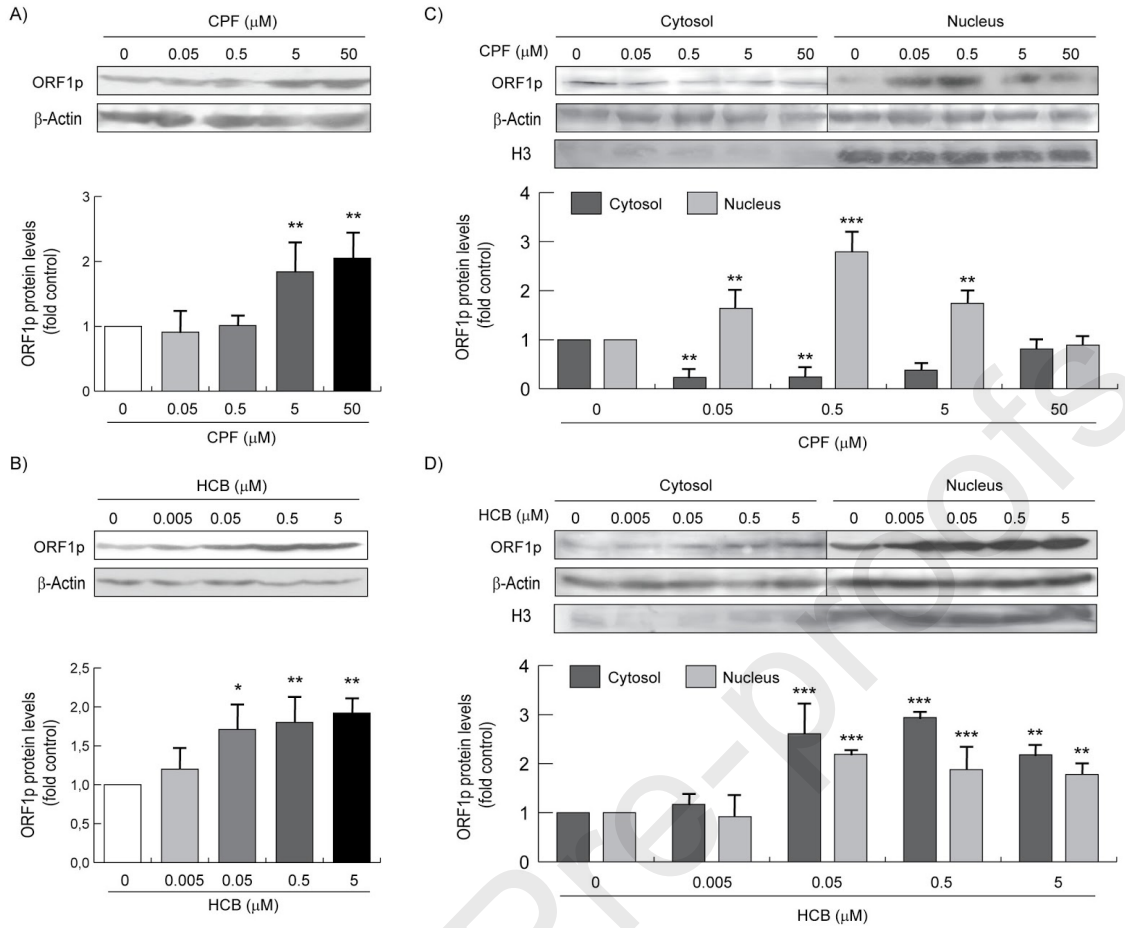

Figure 8: Pesticide action on ORF1p expression and localization in NMuMG cells. (A-B) ORF1p expression levels and (C-D) ORF1p cytoplasmic and nuclear localization. NMuMG cells were exposed to (A, C) CPF (0.05, 0.5, 5, and 50 μM) or (B, D) HCB (0.005, 0.05, 0.5 and 5 μM) for 48 h. Whole cell lysates and cytoplasmic and nuclear fractions were used to analyze ORF1p levels by Western blot. To normalize values, we used anti-β-Actin antibody for whole cell lysates and cytosol, and anti-Histone 3 (H3) for nuclei. A representative Western blot is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p<0.05, **p<0.01, ***p<0.001; ANOVA and Dunnett's post-hoc test).

4.Discussion

Several epigenetic mechanisms can modify genome function under exogenous influence, including the exposure to environmental pollutants acting as EDs [60]. Considering the significant increase in breast cancer incidence observed in recent years and the role of EDs exposure as a potential risk factor [61], studies of epigenetic changes caused by these compounds can provide evidence of how they promote cancer. Abundant research suggests that epigenetic alterations may be one of the mechanisms by which pesticides can have adverse effects on human health [62]. In line with this understanding, the present study demonstrates that environmental relevant concentrations of HCB and CPF enhance LINE-1 expression and ORF1p nuclear import in NMuMG non-tumorigenic mammary epithelial cells. Furthermore, pesticides reduce LINE-1 methylation status and increase LINE-1 activation in the human triple-negative breast cancer cell line MDA-MB-231. These findings may prove crucial, as hypomethylation of LINE-1 correlates with worse prognosis for many types of cancer, such as colorectal, liver, esophageal, breast, bladder, and lung cancers [57]. In particular, LINE-1 expression has been observed in aggressive forms of human breast ER-negative tumors, which exhibit frequent distant metastasis and resistance to hormone therapy [36, 63]. LINE-1 reactivation and subsequent insertion lead to DNA instability, functional knockout of genes, genetic mutations or alterations in gene expression which result in aberrant cellular phenotypes and explain LINE-1 role in carcinogenesis [57, 64]. A direct association has been found between cancer-associated DNA damage and the activation of LINE-1 expression [65]. Accordingly, herein we observed an association between ORF1p nuclear translocation and DNA DSBs, evidenced by the accumulation of γ-H2AX foci after pesticide exposure (0.005 μM HCB and 50 μM CPF). DSBs are generated by exogenous agents or by reactive oxygen species [66]. In this regard, we have previously observed that CPF (50 μM) reduces cell proliferation accompanied by a redox imbalance in this cell line [67] which could contribute to DNA DSBs.

AhR activation has been implicated in the transcriptional and post-transcriptional regulation of LINE-1 [68]. However, some AhR ligands such as 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD) do not induce LINE-1 expression [69]. In this context, the current work shows that the increase induced by CPF or HCB in LINE-1 mRNA levels is mediated by AhR in both cell lines, MDA-MB-231 and NMuMG. A previous investigation conducted in our laboratory found that HCB binds to AhR and triggers c-Src activation [70], which in turn promotes the phosphorylation of the canonical (Smad3) and non-canonical (JNK and p38) TGF-β1 downstream pathways, inducing MDA-MB-231 cell migration and invasion. In addition, AhR activation by HCB leads to TGF-β1 gene expression, which is secreted and accumulated into de culture medium [27]. In a similar way, the current data show that CPF exposure increases AhR protein levels as well as activates c-Src and Smad2/3 in MDA-MB-231 cells, indicating that CPF stimulates AhR/c-Src and TGF-β1 signaling. All findings indicated clearly that both pesticides enhance LINE-1 mRNA expression levels in breast cancer cell line MDA-MB-231, in a dependent manner of the AhR and TGF-β1 signaling. In line with our results, Reyes-Reyes et al. [39] have reported that AhR activation by benzopyrene induces the expression of TGF-β1 and activates TGF-β1/Smad signaling, which subsequently increases LINE-1 mRNA expression. Accordingly, in NMuMG non-tumorigenic mammary epithelial cells, 5 μM CPF heightens LINE-1 mRNA expression through AhR and TGF-β1/Smad signaling. However, CPF induces the LINE-1 mRNA levels at lower doses, which does not alter Smad3 phosphorylation. Besides, 0.005 μM HCB enhances LINE-1 mRNA expression and c-Src activation in NMuMG cells, but without changes in TGF-β1/Smad3 pathway [58], suggesting that AhR/c-Src signaling could be activating other pathways that modulate the LINE-1 mRNA expression.

It has been established that hypomethylation of LINE-1 5´UTR is associated with activation of LINE-1 expression in many types of cancer [71], including breast cancer [30]. Indeed, the degree of LINE-1 hypomethylation increases in more advanced cancers and is also related to tumor size and grade [72]. For instance, HCB promotes mammary tumor growth, angiogenesis and metastasis in different animal models [8], whereas CPF alters HDAC1 mRNA expression

in rat mammary gland and increases tumor incidence in a chemically induced rat mammary tumor model [18]. Therefore, the LINE-1 reactivation induced by the pesticides contributes to explaining how these compounds behave as risk factors for breast cancer progression. However, Ventura et al. [18] noted that CPF treatment does not promote changes in LINE-1 DNA methylation in rat mammary gland, indicating that this CPF action could be specific to breast cancer or that different CpG sites may have been analyzed. In the current study, we found that only the methylation of the first CpG sites evaluated in 5´UTR were reduced by pesticide exposure, in agreement with previous reports [37]. Similar results regarding LINE-1 hypomethylation have been observed after exposure to organochlorine pesticides [73] and other EDs such as phthalate [74]. In addition, placental LINE-1 methylation has been inversely correlated with infant birth length among Korean mothers exposed to persistent organic pollutants [75]. In turn, TGF-β1 signaling has been implicated in the regulation of epigenetic mechanisms including DNA methylation, as Smad proteins interact with chromatin modifying complexes to remodel chromatin structure [76]. Therefore, Smad activation by pesticide exposure could in fact participate in the epigenetic control of the LINE-1.

Hypomethylation of LINE-1 leads to an increase in retrotransposon activity and, consequently, in the translation of ORF1p and ORF2p [57]. However, in the current work, pesticide treatment did not only fail to induce ORF1p expression in MDA-MB-231 cells, but also actually reduced it in the presence of 50 μM CPF. This could be explained by ORF1p degradation mechanism via proteosoma, as deHaro et al. [77] described in different cancer cell lines. Interestingly, in NMuMG mammary epithelial cells, ORF1p expression levels were significantly enhanced by HCB and CPF exposure, in contrast to MDA-MB-231 cells. Rodic et al. [54] have reported that 90% of the breast cancer tissue samples examined were highly positive for ORF1p, while other authors have found high cytoplasmic expression of ORF1p and ORF2p in non-invasive tumors, although this was not related with patient survival [36]. These authors also showed that, for invasive tumors, the nuclear localization of ORF1p and ORF2p was more closely associated with lymph node metastasis and poor patient outcomes than cytoplasmic

expression. Given that ORF1p is required for LINE-1 retrotransposition, changes in the subcellular localization of ORF1p from cytoplasm to nucleus may be a critical step in tumorigenesis. The data presented here show that 0.05-5 μM CPF induced ORF1p nuclear import as well as LINE-1 mRNA expression in NMuMG cell line, while HCB enhanced both, nuclear and cytosolic ORF1p levels at the same doses as the increase in the total protein was observed (0.05-5 μM). On the other hand, in MDA-MB-231 cell line, the lowest HCB dose used (0.005 μM), which is close to current human exposure, induces ORF1p nuclear localization, with similar results observed for 50 μM CPF. Besides, the ORF1p nuclear import after HCB exposure involves AhR and TGF-β1 pathways, while only TGF-β1 signaling is implicated in MDA-MB-231 treated with CPF. It has also been reported that phosphorylation of ORF1p is required for LINE-1 retrotransposition [78]. ORF1p is phosphorylated on multiple serines and threonines and some of these sites are targets for proline-directed protein kinases (PDPKs), a kinase family which includes mitogen-activated protein kinases (MAPKs), cyclin-dependent kinases (CDKs), and glycogen synthase 3 (GSK3) [79]. In this regard, we have shown that HCB increases phosphorylation of ERK1/2 [70], p38 and JNK [27], whereas CPF activates ERK1/2 [67] in MDA-MB-231 cells, findings which could be linked to the increase in LINE-1 retrotransposition induced by the pesticides. Accordingly, Ishizaka et al. [80] have found that strong AhR ligands such as 6-formylindolo[3,2-b]carbazole (FICZ), 3-methylcholantrene and benzopyrene induce LINE-1 retrotransposition mediated via p38 and JNK but not via the classical AhR pathway. In addition, Reyes-Reyes et al. [39] have reported that TGF-β1 pathway mediates LINE-1 retrotransposition in hepatoma cells exposed to benzopyrene. In this line, the current work further shows that LINE-1 reactivation is also induced by weak AhR ligands such as HCB and CPF, and proposes an alternative mechanism of action which involves c-Src phosphorylation, triggering TGF-β1/Smad activation and LINE-1 mRNA expression in MDA-MB-231 breast cancer cells (Fig. 9A). Similar results were obtained in NMuMG mammary epithelial cells exposed to CPF, however in the presence of HCB, LINE-1 mRNA levels are regulated by a TGF-β1/Smad independent mechanism (Fig. 9B).

In sum, our results provide experimental evidence that pesticide exposure may modify LINE-1 methylation levels and induce LINE-1 reactivation, suggesting that epigenetic mechanisms could contribute to pesticide-induced breast cancer progression.

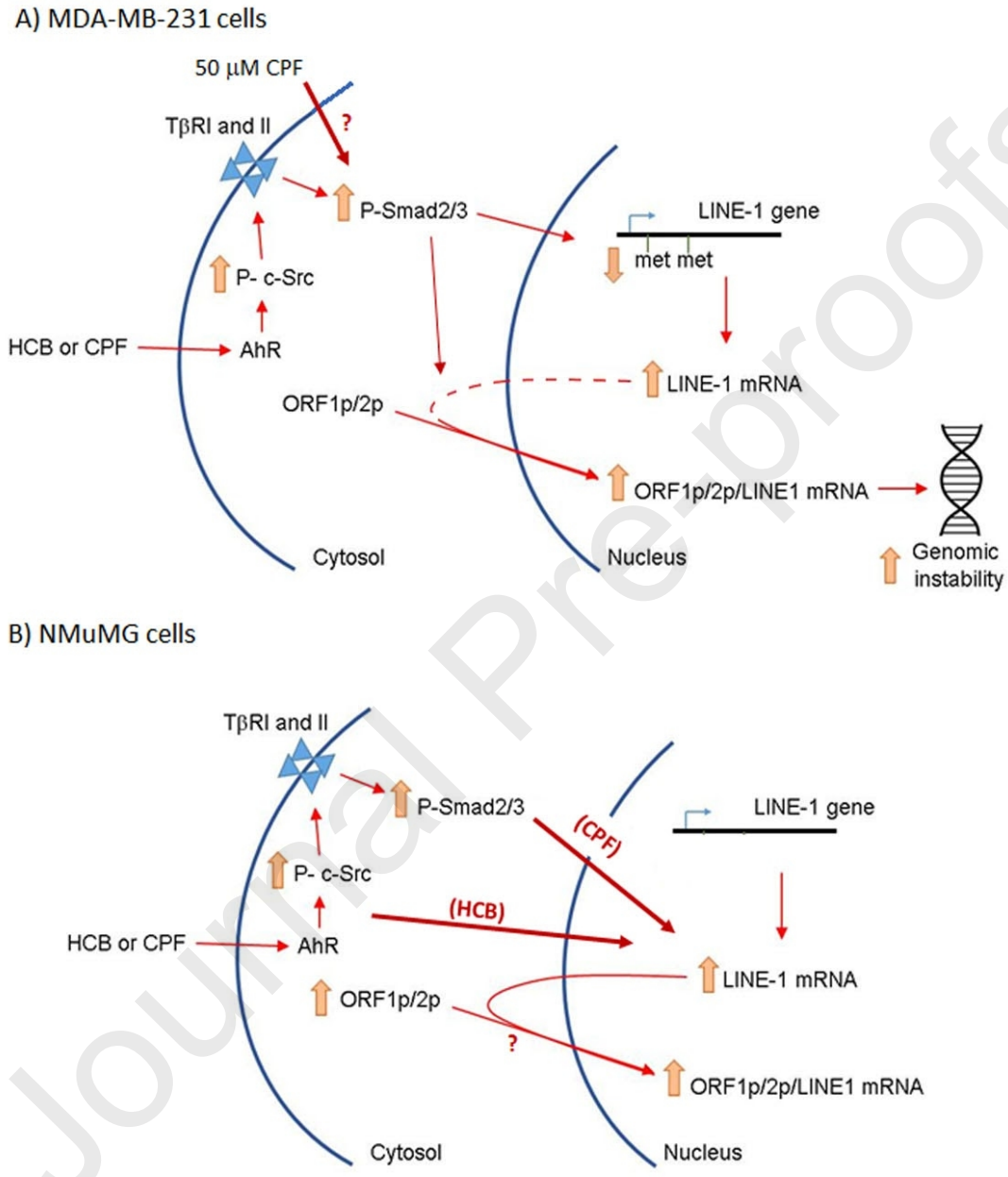

Fig. 9. Model depicting molecular mechanisms of CPF and HCB action on LINE-1 reactivation. (A) MDA-MB-231 cells: The pesticide (CPF or HCB) binds to AhR/c-Src complex and triggers c-Src activation, raising the phosphorylation of TGF-β1 canonical downstream pathway (Smad2/3). This effect results in the demethylation of

the LINE-1 internal promoter and leads the LINE-1 transcription, but without great changes in ORF1p expression. Then, ORF1p (possible forming a complex with ORF2p and LINE-1 mRNA) is imported to the nucleus by a mechanism that involves TGF-β1/Smad signaling. The ORF1p translocation to the nucleus after 50 μM CPF treatment is AhR-independent, suggesting that CPF could be activating Smad by an unknown mechanism (?). Finally, the LINE-1 reactivation along with other mechanisms could lead to an increase in genomic instability. (B) NMuMG cells: CPF activates AhR/c-Src and TGF-β1/Smad3 pathways, which results in LINE-1 transcription. HCB also enhances LINE-1 mRNA levels in an AhR-dependent manner but without involve TGF-β1/Smad3 signaling. In addition, both pesticides increase ORF1p expression and nuclear localization.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

References

[1] R.E. Ellsworth, P.J. Kostyniak, L.H. Chi, C.D. Shriver, N.S. Costantino, D.L. Ellsworth, Organochlorine pesticide residues in human breast tissue and their relationships with clinical and pathological characteristics of breast cancer, EnvironToxicol, 33 (2018), pp. 876–884.

[2] C. Fenga, Occupational exposure and risk of breast cancer, Biomed Rep, 4 (3) (2016), pp. 282-292.

[3] R.E. Bailey, Global hexachlorobenzene emissions, Chemosphere, 43 (2) (2001), pp. 167-182.

[4] N. Bravo, S. Hansen, I. Økland, M. Garí, M.V. Álvarez, S. Matiocevich, J.Ø. Odland, J.O. Grimalt, Influence of maternal and sociodemographic characteristics on the accumulation of organohalogen compounds in Argentinian women. The EMASAR study. Environ Res, 158 (2017), pp. 759–767.

[5] X. Chen, P. Panuwet, R.E. Hunter, A.M. Riederer, G.C. Bernoudy, D.B. Barr, P.B. Ryan, Method for the quantification of current use and persistent pesticides in cow milk, human milk and baby formula using gas chromatography tandem mass spectrometry, J. Chromatogr. B Anal Technol Biomed Life Sci, 970 (2014), pp. 121–130.

[6] H. Guo, Y. Jin, Y. Jin, Y. Cheng, B. Leaderer, S. Lin, T.R. Holford, J. Qiu, Y. Zhang, K. Shi, Y. Zhu, J. Niu, B.A. Bassig, S. Xu, B. Zhang, Y. Li, X. Hu, Q. Chen, T. Zheng, Prenatal exposure to organochlorine pesticides and infant birth weight in China, Chemosphere, 110 (2014), pp. 1–7.

[7] M. Harmouche-Karaki, J. Matta, K. Helou, Y. Mahfouz, N. Fakhoury-Sayegh, J.F. Narbonne, Serum concentrations of selected organochlorine pesticides in a Lebanese population and their associations to sociodemographic, anthropometric and dietary factors: ENASB study, Environ Sci Pollut Res Int, 25 (15) (2018), pp. 14350–14360.

[8] N.V. Miret, C.A. Pontillo, L.V. Zárate, D. Kleiman de Pisarev, C. Cocca, A.S. Randi, Impact of endocrine disruptor hexachlorobenzene on the mammary gland and breast cancer: The story thus far, Environm Res, 173 (2019), pp. 330–341.

[9] F. Chiappini, L. Alvarez, V. Lux-Lantos, A.S. Randi, D.L. Kleiman de Pisarev, Hexachlorobenzene triggers apoptosis in rat thyroid follicular cells. Toxicol Sci, 108 (2) (2009), pp. 301–310.

[10] D. Peña, C. Pontillo, M.A. García, C. Cocca, L. Alvarez, F. Chiappini, N. Bourguignon, I. Frahm, R. Bergoc, D.L. Kleiman de Pisarev, A. Randi, Alterations in c-Src/HER1 and Estrogen Receptor a signaling pathways in mammary gland and tumors of Hexachlorobenzene-treated rats. Toxicology, 293 (2012), pp. 68–77.

[11] Agency for Toxic Substances and Disease Registry, ATSDR, (2015), Toxicological Profile for Hexachlorobenzene. http://dx.doi.org/10.1155/2013/286524.

[12] S. Jergentz, H. Mugni, C. Bonetto, R. Schulz R, Assessment of insecticide contamination in runoff and stream water of small agricultural streams in the main soybean area of Argentina. Chemosphere, 61 (6) (2005), pp. 817–826.

[13] R.M. Loewy, L.B. Monza, V.E. Kirs, M.C. Savini, Pesticide distribution in an agricultural environment in Argentina. J Environ Sciense Heal, 46 (8) (2011), pp. 662–670.

[14] K. Huen, A. Bradman, K. Harley, P. Yousefi, D. Boyd Barr, B. Eskenazi, N. Holland. Organophosphate pesticide levels in blood and urine of women and newborns living in an agricultural community. Environ Res. 117 (2012), pp. 8–16.

[15] S. Srivastava, S.S. Narvi, S.C. Prasad. Level of select organophosphates in human colostrums and mature milk sample in rural region of Faizabad district, Uttar Pradesh, India. Hum Exp Toxicol, 30 (2011), pp. 1458-1463.

[16] A.J. Li, K. Kannan, Urinary concentrations and profiles of organophosphate and pyrethroid pesticide metabolites and phenoxyacid herbicides in populations in eight countries. Environ Int, 121 (Pt 2) (2018), pp. 1148-1154.

[17] C. Ventura, M.R. Nieto, N. Bourguignon, V. Lux-Lantos, H. Rodriguez, G. Cao, A. Randi, C. Cocca, M. Núñez, Pesticide chlorpyrifos acts as an endocrine disruptor in adult rats causing changes in mammary gland and hormonal balance. J Steroid Biochem Mol Biol, 156 (2016), pp. 1-9.

[18] C. Ventura, C.D. Zappia, M. Lasagna, W. Pavicic, S. Richard, A.D. Bolzan, F. Monczor, M. Núñez, C. Cocca, Effects of the pesticide chlorpyrifos on breast cancer disease. Implication of epigenetic mechanisms. J Steroid Biochem Mol Biol, 186 (2019), pp. 96-104.

[19] M.E. Hahn, J.A. Goldstein, P. Linko, T.A. Gasiewicz, Interaction of Hexachlorobenzene with the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin in vitro and in vivo. ArchBiochemBiophys, 270 (1989), pp. 344–355.

[20] S. Medjakovic, A. Zoechling, P. Gerster, M.M. Ivanova, Y. Teng, C.M. Klinge, B. Schildberger, M. Gartner, A. Jungbauer, Effect of non persistent pesticides on estrogen receptor, androgen receptor, and aryl hydrocarbon receptor. Environ Toxicol, 29 (2014), pp. 1201-1216.

[21] S. Park, B. Dong, F. Matsumura, Rapid activation of c-Src kinase by dioxin is mediated by the Cdc37-HSP90 complex as part of Ah receptor signaling in MCF10A cells. Biochemistry, 46 (2007), pp. 899–908.

[22] F. Matsumura, How important is the protein phosphorylation pathway in the toxic expression of dioxin-type chemicals. Biochem Pharmacol, 48 (1994), pp. 215–24.

[23] L.A. Torre, R.L. Siegel, E.M. Ward, A. Jemal, Global cancer incidence and mortality rates and trends-an update. Cancer Epidemiol Biomarkers Prev, 25 (2016), pp. 16–27.

[24] H. Moses, M.H. Barcellos-Hoff, TGF-β biology in mammary development and breast cancer. Cold Spring Harb Perspect Biol, 3 (2011), pp. 1–14.

[25] X. Chang, Y. Fan, S. Karyala, S. Schwemberger, C.R. Tomlinson, M.A. Sartor, and A. Puga, Ligand-Independent Regulation of Transforming Growth Factor-β1 Expression and Cell Cycle Progression by the Aryl Hydrocarbon Receptor, Mol and Cell Biology, 27 (17) (2007), pp. 6127–6139.

[26] E.M. Rico-Leo, A. Alvarez-Barrientos, P.M. Fernandez-Salguero, Dioxin receptor expression inhibits basal and transforming growth factor β-induced epithelial-to-mesenchymal transition. J Biol Chem, 288 (11) (2013), pp. 7841-56.

[27] N.V. Miret, C. Pontillo, C. Ventura, A. Carozzo, F. Chiappini, D. Kleiman de Pisarev, N. Fernández, C. Cocca, A. Randi, Hexachlorobenzene modulates the crosstalk between the

Aryl Hydrocarbon Receptor and Transforming Growth Factor-β1 signaling, enhancing human breast cancer cell migration and invasion. Toxicology, 366 (2016), pp. 20–31.

[28] N.V. Miret, E. Rico-Leo, C. Pontillo, E. Zotta, P. Fernández-Salguero, A. Randi. A dioxin-like compound induces hyperplasia and branching morphogenesis in mouse mammary gland, through alterations in TGF-β1 and aryl hydrocarbon receptor signaling. Toxicol. Appl. Pharmacol. 334 (2017), pp. 192–206.

[29] A. Baccarelli, V. Bollati, Epigenetics and environmental chemicals. Curr Opin Pediatr, 21 (2009), pp. 243–251.

[30] S.Y. Park, A.N. Seo, H.Y. Jung, J.M. Gwak, N. Jung, N.Y. Cho, G.H. Kang, Alu and LINE-1 hypomethylation is associated with HER2 enriched subtype of breast cancer. PLoS One, 9 (6) (2014), Article e100429.

[31] C.R. Beck, J.L. Garcia-Perez, R.M. Badge, J.V. Moran, LINE-1 elements in structural variation and disease. Annu Rev Genomics Hum Genet, (12) (2011), pp. 187-215.

[32] I. Sciamanna, C. De Luca, C. Spadafora, The Reverse Transcriptase Encoded by LINE-1 Retrotransposons in the Genesis, Progression, and Therapy of Cancer. Front Chem, 11 (4) (2016), p. 6.

[33] J.M.C. Tubio, Y. Li, Y.S. Ju, I. Martincorena, S.L. Cooke, M. Tojo, G. Gundem, C.P. Pipinikas, J. Zamora, K. Raine K, et al., Mobile DNA in cancer. Extensive transduction of nonrepetitive DNA mediated by L1 retrotransposition in cancer genomes. Science, 345 (6196) (2014), Article 1251343.

[34] R. Cordaux, M.A. Batzer, The impact of retrotransposons on human genome evolution. Nat Rev Genet, 10 (10) (2009), pp. 691-703.

[35] S.M. Belgnaoui, R.G. Gosden, O.J. Semmes, A. Haoudi, Human LINE-1 retrotransposon induces DNA damage and apoptosis in cancer cells. Cancer Cell International, 6 (2006), pp. 13.

[36] S. Burma, B.P. Chen, M. Murphy, A. Kurimasa, D.J. Chen, ATM phosphorylates histone H2AX in response to DNA double-strand breaks. J Biol Chem, 276(45) (2001), pp. 42462-42467.

[37] K. Hata, Y. Sakaki, Identification of critical CpG sites for repression of L1 transcription by DNA methylation. Gene 189 (1997), pp. 227–234.

[38] L. Chen, J.E. Dahlstrom, A. Chandra, P. Board, D. Rangasamy, Prognostic value of LINE-1 retrotransposon expression and its subcellular localization in breast cancer. Breast Cancer Res Treat, 136 (2012), pp. 129-142.

[39] E.M. Reyes-Reyes, I.N. Ramos, M.A. Tavera-García, K.S. Ramos, The aryl hydrocarbon receptor agonist benzo(a)pyrene reactivates LINE-1 in HepG2 cells through canonical TGF-β1 signaling: implications in hepatocellular carcinogenesis. Am J Cancer Res, 6 (2016), pp. 1066-1077.

[40] M.A. García, D. Peña, L. Alvarez, C. Cocca, C. Pontillo, R. Bergoc, D. Kleiman de Pisarev, A. Randi, Hexachlorobenzene induces cell proliferation and IGF-I signaling pathway in an estrogen receptor alpha-dependent manner in MCF-7 breast cancer cell line, Toxicol. Lett, 192 (2010), pp. 195–205.

[41] J. To- Figueras, M. Sala, R. Otero, C. Barrot, M. Santiago-Silva, M. Rodamilans, C. Herrero, J. Grimalt, J. Sunyer, Metabolism of hexachlorobenzene in humans: association between serum levels and urinary metabolites in a highly exposed population. Environ. Health Perspect, 105 (1997), pp. 78–83.

[42] A. Saoudi, N. Fréry, A. Zeghnoun, M.L. Bidondo, V. Deschamps, T. Göen, R. Garnier, L. Guldner, Serum levels of organochlorine pesticides in the French adult population: the French national nutrition and health study (ENNS), 2006–2007. Sci. Total Environ,472 (2014), pp. 1089–1099.

[43] L.L. Cao, C.H. Yan, X.D. Yu, Y. Tian, L. Zhao, J.X. Liu, X.M. Shen, Relationship between serum concentrations of polychlorinated biphenyls and organochlorine pesticides and dietary habits of pregnant women in Shanghai. Sci Total Environ, 2011 (2011), pp. 2997–3002.

[44] K. Becker, S. Kaus, C. Krause, P. Lepom, C. Schulz, M. Seiwert, B. Seifert, German Environmental Survey 1998 (GerES III): environmental pollutants in blood of the German population, Int J Hyg Environ Health, 205 (2002), pp. 297–308.

[45] K. Harnpicharnchai, N. Chaiear, L. Charerntanyarak L, Residues of organophosphate pesticides used in vegetabla cultivation in ambient air, surface water and soil in Bueng Niam Subdistrict, Khon Kaen, Thailand, Southeast Asian. J Trop Med Public Health, 44 (2013), pp. 1088–1097.

[46] R.A. Jensen, D.L. Page, W.D. Dupont, L.W. Rogers, Invasive breast cancer risk in women with sclerosing adenosis. Cancer, 64(1989), pp.1977–1983.

[47] R. Sanghi, M.K.K. Pillai, T.R. Jayalekshmi, A. Nair. Organochlorine and organophosphorus pesticide residues in breast milk from Bhopal, Madhya Pradesh, India. Hum Exp Toxicol, 22 (2) (2003), pp. 73-76.

[48] F. Chiappini, C. Pontillo, A. Randi, L. Alvarez, D.L. Kleiman de Pisarev, Hexachlorobenzene induces TGF-b1 expression, which is a regulator of p27 and cyclin D1 modifications. Toxicology Lett, 230 (2014), pp. 1–9.

[49] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem, 72 (1976), pp. 248–254.

[50] A.L. Gomez, G.A. Altamirano, J. Leturia, V.L. Bosquiazzo, M. Muñoz-de-Toro, L. Kass, Male mammary gland development and methylation status of estrogen receptor alpha in Wistar rats are modified by the developmental exposure to a glyphosate-based herbicide. Mol Cell Endocrinol, 481 (2019), pp. 14-25.

[51] Š. Čikoš, A. Bukovská, J. Koppel, Relative quantification of mRNA: comparison of methods currently used for real-time PCR data analysis, BMC Mol Biol, 8 (2007), pp. 113.

[52] A.Q. van Hoesel, C.J. van de Velde, P.J. Kuppen, G.J. Liefers, H. Putter, Y. Sato, D.A. Elashoff, R.R. Turner, J.M. Shamonki, E.M. de Kruijf, J.G. van Nes, A.E. Giuliano, D.S. Hoon, Hypomethylation of LINE-1 in primary tumor has poor prognosis in young breast cancer patients: a retrospective cohort study. Breast Cancer Res Treat, 134 (3) (2012), pp. 1103-1114.

[53] K. Hata, Y. Sakaki, Identification of critical CpG sites for repression of L1 transcription by DNA methylation, Gene, 189 (1997), pp. 227–234.

[54] N. Rodić, R. Sharma, R. Sharma, J. Zampella, L. Dai, M.S. Taylor, R.H. Hruban, C.A. Iacobuzio-Donahue, A. Maitra, M.S. Torbenson, et al., Long interspersed element-1 protein expression is a hallmark of many human cancers, Am J Pathol, 184 (5) (2014), pp. 1280-1286.

[55] S.L. Martin, The ORF1 protein encoded by LINE-1: Structure and function during L1 retrotransposition. J Biomed Biotechnol, 1 (2006), pp. 45621.

[56] D.R. Pilch, O.A. Sedelnikova, C. Redon, A. Celeste, A. Nussenzweig, W.M. Bonner, Characteristics of gamma-H2AX foci at DNA double-strand breaks sites. Biochem Cell Biol, 81(3) (2003), pp. 123-129.

[57] A. Lavasanifar, C.N. Sharp, E.A. Korte, T. Yin, K. Hosseinnejad, S.A. Jortani, Long interspersed nuclear element-1 mobilization as a target in cancer diagnostics, prognostics and therapeutics. Clin Chim Acta, 493 (2019), pp. 52–62.

[58] N.V. Miret, Doctoral Thesis. Acción de disruptores endocrinos en cáncer de mama. Rol del factor de crecimiento transformante-β 1 y del receptor de hidrocarburos aromáticos. (PhD thesis. Universidad de Buenos Aires. Facultad de Ciencias Exactas y Naturales) (2018). http://hdl.handle.net/20.500.12110/tesis_n6288_Miret.

[59] I. Sciamanna, M. Landriscina, C. Pittoggi, M. Quirino, C. Mearelli, R. Beraldi, E. Mattei, A. Serafino, A. Cassano, P. Sinibaldi-Vallebona, E. Garaci, C. Barone, C. Spadafora, Inhibition of endogenous reverse transcriptase antagonizes human tumor growth, Oncogene, 24 (24) (2005), pp. 3923–3931.

[60] A. Alavian-Ghavanini, J. Rüegg, Understanding epigenetic effects of endocrine disrupting chemicals: from mechanisms to novel test methods. Basic Clin Pharmacol Toxicol, 122 (1) (2018), pp. 38-45.

[61] IBCERCC, Report of the Interagency Breast Cancer and Environmental Research Coordinating Committee. Breast Cancer and the Environment: Prioritizing Prevention (2013).

[62] M. Collotta, P.A. Bertazzi, V. Bollati, Epigenetics and pesticides. Toxicology, 307 (2013), pp. 35-41.

[63] C.R. Harris, R. Normart, Q. Yang, E. Stevenson, B.G. Haffty, S. Ganesan, C. Cordon-Cardo, A.J. Levine, L.H. Tang, Association of nuclear localization of a long interspersed

nuclear element-1 protein in breast tumors with poor prognostic outcomes. Genes Cancer, 1 (2) (2010), pp. 115-124.

[64] S. Ohms, S.H. Lee and D. Rangasamy, LINE-1 retrotransposons and let-7 miRNA: partners in the pathogenesis of cancer? Front Genet, 5 (2014), pp. 338.

[65] N.A. Wallace, V.P. Elancio, Z. Faber, and P. Deininger, Feed-back inhibition of L1 and alu retrotransposition through altered double strand break repair kinetics. Mob DNA, 1 (2010), pp. 22.

[66] K.K. Khanna, S.P. Jackson, DNA double-strand breaks: signaling, repair and the cancer connection. Nat Genet, 27 (2001) (3), pp. 247-54.

[67] C. Ventura, A. Venturino, N. Miret, A. Randi, E. Rivera, M. Núñez, C. Cocca, Chlorpyrifos inhibits cell proliferation through ERK1/2 phosphorylationin breast cancer cell lines. Chemosphere, 120 (2015), pp. 343–350.

[68] N. Okudaira, T. Okamura, M. Tamura, K. Iijma, M. Goto, A. Matsunaga, M. Ochiai, H. Nakagama, S. Kano, Y. Fujii-Kuriyama, Y. Ishizaka, Long interspersed element-1 is differentially regulated by food-borne carcinogens via the aryl hydrocarbon receptor, Oncogene, 32 (41) (2013), pp. 4903-4912.

[69] B.E. McIntosh, J.B. Hogenesch, and C.A. Bradfield, Mammalian Per-Arnt-Sim proteins in environmental adaptation. Ann. RevPhysiol, 72 (2010), pp. 625–645.

[70] C. Pontillo, M.A. García, D. Peña, C. Cocca, F. Chiappini, L. Alvarez, D. Kleiman de Pisarev, A. Randi, Activation of c-Src/HER1/STAT5b and HER1/ERK1/2 signaling pathways and cell migration by hexachlorobenzene in MDA-MB-231 human breast cancer cell line. Toxicol Sci, 120 (2011), pp. 284–296.

[71] H.A. Cruickshanks, C. Tufarelli, Isolation of cancer- specific chimeric transcripts induced by hypomethylation of the LINE-1 antisense promoter. Genomics, 94 (2009), pp. 397–406.

[72] N. Kitkumthorn, A. Mutirangura, Long interspersed nuclear element-1 hypomethylation in cancer: biology and clinical applications. Clin Epigenet, 2 (2011), pp.315–330.

[73] K.Y. Kim, D.S. Kim, S.K. Lee, I.K. Lee, J.H. Kang, Y.S. Chang, D.R. Jacobs, M. Steffes, D.H. Lee, Association of low-dose exposure to persistent organic pollutants with global DNA hypomethylation in healthy Koreans. Environ Health Perspect, 118 (2010), pp. 370–374.

[74] L. Montrose, V. Padmanabhan, J.M. Goodrich, S.E. Domino, M.C. Treadwell, J.D. Meeker, D.J. Watkins, D.C. Dolinoy, Maternal levels of endocrine disrupting chemicals in the first trimester of pregnancy are associated with infant cord blood DNA methylation. Epigenetics, 13(3) (2018), pp. 301-309.

[75] S. Kim, Y.H. Cho, I. Lee, W. Kim, S. Won, J.L. Ku, H.B. Moon, J. Park, S. Kim, G. Choi, K. Choi, Prenatal exposure to persistent organic pollutants and methylation of LINE-1 and imprinted genes in placenta: A CHECK cohort study. Environ Int, 119(2018), pp. 398-406.

[76] J. Massague, TGFbeta signalling in context. Nat Rev Mol Cell Biol, 13 (2012), pp. 616-630.

[77] D. deHaro, K.J. Kines, M. Sokolowski, R.T. Dauchy, V.A. Streva, S.M. Hill, J.P. Hanifin, G.C. Brainard, D.E Blask, V.P. Belancio. Regulation of L1 expression and retrotransposition by melatonin and its receptor: implications for cancer risk associated with light exposure at night. Nucleic Acids Res. 42(12) (2014), pp. 7694-707.

[78] P.R. Cook, C.E. Jones, A.V. Furano. Phosphorylation of ORF1p is required for L1 retrotransposition. PNAS, 112 (2015), pp. 4298–4303.

[79] A.V. Furano, P.R. Cook, The challenge of ORF1p phosphorylation: Effects on L1 activity and its host. Mob Genet Elements, 6 (1) (2015), Article e1119927.

[80] Y. Ishizaka, N. Okudaira, T. Okamura. Regulation of retrotransposition of long interspersed element-1 by mitogen-activated protein kinases. Protein Kinases, ed G. Da Silva Xavier DGDS (InTech, Rijeka, Croatia). (2012), Available from: http://www.intechopen.com/books/proteinkinases/regulation-of-retrotransposition-of-long-interspersed-element-1-by-mitogen-activated-protein-kin.

Figure and table legends

Table 1. Primer sequences for qPCR analyses.

Fig. 1. HCB and CPF action on LINE-1 mRNA expression levels in MDA-MB-231 cells. LINE-1 ORF1 and ORF2 mRNA levels were evaluated by RT-qPCR. Cells were exposed to (A) CPF (0.05, 0.5, 5, and 50 μM) for 48 h or (B) HCB (0.005, 0.05, 0.5, and 5 μM) for 24 h. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (**p<0.01 and ***p<0.001; ANOVA and Dunnett's post hoc test).

Fig. 2. Pesticide effects on LINE-1 methylation status in MDA-MB-231 cells. (A) Map of LINE-1 5´-UTR and its CpG island. Target sites for digestion by HpaII, BstUI and HinfI methylation-sensitive restriction enzymes are shown. Positions of PCR primers and their amplification products are indicated by arrows and lines, respectively. IC: internal control region. We named 1 to the first nucleotide from 5´UTR. (B-G) Cells were exposed to 0.5 μM CPF for 48 h, 0.005 μM HCB for 24 h or vehicle. Graphs show the relative methylation status of (B) HpaII a site (87), (C) HpaII b site (152), (D) BstUI site (216), (E) HinfI a site (283), (F) HpaII c site (355), and (G)HinfI b site (422). Values are expressed as the mean ± SEM of at least three independent experiments (*p< 0.05, Mann Whitney U test).

Fig. 3. CPF action on AhR and TGF-β1 signaling pathways in MDA-MB-231 cells. (A) AhR, (B-C) phospho (P)- and total-c-Src, and (D-E) P- and total-Smad2 and 3 protein levels.

(A, D) Dose-response studies: cells were exposed to CPF (0.05, 0.5, 5, and 50 μM CPF) or vehicle for (A) 24 h or (C) 15 min. (B, E) Time-course studies: cells were treated with CPF (0.5 μM) or vehicle for 5, 15 and 30 min, 2 and 6 h. (C) Cells were pretreated with PHE (5 μM) or vehicle (DMSO) for 1 h and then treated with CPF (0.5 μM) in the presence or absence of inhibitor for 15 min. Whole-cell lysates were used to analyze protein levels by Western blot. The AhR protein/β-Actin protein ratio or phosphorylated protein/total protein ratio were normalized to control values. A representative Western blot from at least three independent experiments is shown in the upper panels (Smad2 top band, Smad3 lower band). Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p < 0.05, **p < 0.01 and ***p < 0.001; ANOVA and Dunnett's post-hoc test).

Fig. 4: Pesticide-induced LINE-1 expression depends on AhR and TGF-β1 signaling. Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle (DMSO) for 1 h and then treated with (A) CPF (0.5 μM) for 48 h or (B) HCB (0.005 μM) for 24 h, in the presence or absence of the inhibitors. LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (***p<0.001) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

Fig. 5. Pesticide effects on ORF1p protein expression and localization in MDA-MB-231 cells. (A-B) ORF1p expression and (C-D) ORF1p cytoplasmic and nuclear localization and (E-F) ORF1p nuclear translocation analyzed by Western blot. Cells were exposed to (A, C) CPF (0.05, 0.5, 5, and 50 μM) or EtOH for 48 h, and (B, D) HCB (0.005, 0.05, 0.5 and 5 μM) or EtOH for 24 h. (E-F) Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle

(DMSO) for 1 h and then exposed to (E) CPF (50 μM) for 48 h or (F) HCB (0.005 μM) for 24 h, in the presence of inhibitors. To normalize values, we used (A-B) anti-β-Actin antibody for whole cell lysates, (C-F) anti-Histone 3 (H3) for nuclei and (C-D) anti-GAPDH for cytosol. A representative Western blot is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p< 0.05 and **p< 0.01; ANOVA and Dunnett's post-hoc test) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

Figure 7: HCB and CPF modulate LINE-1 mRNA expression in non-tumorigenic mammary epithelial cells NMuMG. Role of AhR and TGF-β1 signaling. Cells were exposed to (A) CPF (0.05, 0.5, 5, and 50 μM) or (B) HCB (0.005, 0.05, 0.5, and 5 μM) for 48 h and LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. β2-microglobulin (β2-M) expression was used as a control to normalize the data. (C) Phospho (P)- c-Src, and P- Smad3 protein levels were determined by Western blot after 15 min of CPF treatment (0.05, 0.5, 5, and 50 μM). Values were normalized by immunoblotting using anti-β-Actin antibody. A Western blot from one representative experiment is shown in the upper panel. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. (D-E) Cells were pretreated with SB431542 (2 μM), PHE (5 μM) or vehicle (DMSO) for 1 h and then exposed to (D) CPF (5 μM) or (E) HCB (0.005 μM) for 48 h, in the presence of the inhibitor. LINE-1 ORF1 mRNA levels were evaluated by RT-qPCR. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs. control (*p<0.05, **p<0.01, ***p<0.001; ANOVA and Dunnett's post-hoc test) and crosses indicate significant differences vs. pesticide treatment (+p<0.05, ++p<0.01 and +++p<0.001; ANOVA and Tukey post hoc test).

Figure 8: Pesticide action on ORF1p expression and localization in NMuMG cells. (A-B) ORF1p expression levels and (C-D) ORF1p cytoplasmic and nuclear localization. NMuMG cells were exposed to (A, C) CPF (0.05, 0.5, 5, and 50 μM) or (B, D) HCB (0.005, 0.05, 0.5 and 5 μM) for 48 h. Whole cell lysates and cytoplasmic and nuclear fractions were used to analyze ORF1p levels by Western blot. To normalize values, we used anti-β-Actin antibody for whole cell lysates and cytosol, and anti-Histone 3 (H3) for nuclei. A representative Western blot is shown in the upper panels. Quantification by densitometry scanning of the immunoblots is shown in the lower panels. Values are expressed as the mean ± SD of at least three independent experiments. Asterisks indicate significant differences vs control (*p<0.05, **p<0.01, ***p<0.001; ANOVA and Dunnett's post-hoc test).

Fig. 9. Model depicting molecular mechanisms of CPF and HCB action on LINE-1 reactivation. (A) MDA-MB-231 cells: The pesticide (CPF or HCB) binds to AhR/c-Src complex and triggers c-Src activation, raising the phosphorylation of TGF-β1 canonical downstream pathway (Smad2/3). This effect results in the demethylation of the LINE-1 internal promoter and leads the LINE-1 transcription, but without great changes in ORF1p expression. Then, ORF1p (possible forming a complex with ORF2p and LINE-1 mRNA) is imported to the nucleus by a mechanism that involves TGF-β1/Smad signaling. The ORF1p translocation to the nucleus after 50 μM CPF treatment is AhR-independent, suggesting that CPF could be activating Smad by an unknown mechanism (?). Finally, the LINE-1 reactivation along with other mechanisms could lead to an increase in genomic instability. (B) NMuMG cells: CPF activates AhR/c-Src and TGF-β1/Smad3 pathways, which results in LINE-1 transcription. HCB also enhances LINE-1 mRNA levels in an AhR-dependent manner but without involve TGF-β1/Smad3 signaling. In addition, both pesticides increase ORF1p expression and nuclear localization.

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Name	**Sense (5'-3')**	**Antisense (5'-3')**	**Product size**
HpaII (a)	GAGGAGCCAAGATGGCCGAA	AATCACCGTCTTCTGCGTCG	75 pb
HpaII (b)	GTGAGCGACGCAGAAGACGG	CCCACTGTCTGGCACTCCCT	82 pb
HpaII (c) and IC (2)	GTCGCACCTGGAAAATCGGG	CCGAGCCAGGTGTGGGATAT	101 pb
BstUI /HinfI(a) and IC (1)	GTGGGCGCAGGCCAGTGTGT	TCCAGGTGCGACCGTCACCC	133 pb
HinfI (b)	TTAAGAAACGGCGCACCACG	GCCGCCTTGCAGTTTGATCT	113 pb
LINE-1 ORF1	CAAGTTGGAAAACACTCTGCAG	GGAGTATCTTTGTGGCGTTCT	109 pb
LINE-1 ORF2	TCGACACATACACTCTCCCAAG	GCCACAATTTCAGAGCCTGTT	82 pb
Human β-Actin	GGACTTCGAGCAAGAGATGG	AGCACTGTGTTGGCGTACAG	234 pb
Mouse β2-Microglobulin	CAAGTATACTCACGCCACCCA	GCAGGCGTATGTATCAGTCTC	219 pb