All trans-retinoic acid analogs promote cancer cell apoptosis through non-genomic Crabp1 mediating ERK1/2 phosphorylation

Shawna D. Persaud¹,*, Sung Wook Park¹,*, Mari Ishigami-Yuasa², Naoko Koyano-Nakagawa³, Hiroyuki Kagechika² & Li-Na Wei¹

All trans-retinoic acid (atRA) is one of the most potent therapeutic agents, but extensive toxicity caused by nuclear RA receptors (RARs) limits its clinical application in treating cancer. AtRA also exerts non-genomic activities for which the mechanism remains poorly understood. We determine that cellular retinoic acid binding protein 1 (Crabp1) mediates the non-genomic activity of atRA, and identify two compounds as the ligands of Crabp1 to rapidly and RAR-independently activate extracellular signal regulated kinase 1/2 (ERK1/2). Non-canonically activated ERK activates protein phosphatase 2A (PP2A) and lengthens cell cycle duration in embryonic stem cells (ESC). This is abolished in Crabp1-null ESCs. Re-expressing Crabp1 in Crabp1-negative cancer cells also sensitizes their apoptotic induction by atRA. This study reveals a physiological relevance of the non-genomic action of atRA, mediated by Crabp1, in modulating cell cycle progression and apoptosis induction, and provides a new cancer therapeutic strategy whereby compounds specifically targeting Crabp1 can modulate cell cycle and cancer cell apoptosis in a RAR-independent fashion, thereby avoiding atRA's toxicity caused by its genomic effects.
The non-genomic effects of atRA are particularly interesting in a stem cell context such as embryonic stem cells (ESCs) and cancer cells. Maintaining their self-renewal requires a tight control over cell cycle progression that ultimately governs cell proliferation, differentiation, senescence and apoptosis. It is known that during differentiation protein phosphatase 2A (PP2A) activity gradually increases; and inhibiting PP2A promotes ESC self-renewal. Interestingly, in this current study we identify PP2A as a target of non-genomic ERK1/2 activation, elicited by holo-Crabp1, in ESC.

From a translational point of view, atRA can be a potent therapeutic for various diseases because of its anti-proliferative, anti-oxidative, pro-apoptotic, and differentiation-inducing activities. It has been most successful in treating acute promyelocytic leukemia. In animal models of skin, oral, lung, breast, bladder, ovarian, and prostate cancers, atRA has also been found to suppress carcinogenesis. Interestingly, in this current study we identify PP2A as a target of non-genomic ERK1/2 activation, elicited by holo-Crabp1, in ESC.

From a translational point of view, atRA can be a potent therapeutic for various diseases because of its anti-proliferative, anti-oxidative, pro-apoptotic, and differentiation-inducing activities. It has been most successful in treating acute promyelocytic leukemia. In animal models of skin, oral, lung, breast, bladder, ovarian, and prostate cancers, atRA has also been found to suppress carcinogenesis. Interestingly, in this current study we identify PP2A as a target of non-genomic ERK1/2 activation, elicited by holo-Crabp1, in ESC.

Results

Ligands for Crabp1 induce rapid, RAR-independent activation of ERK1/2. Retinoids are structurally comprised of a bulky hydrophobic region, a terminal polar functional group (carboxyl group or its bioisosteres), and the linker between them. We screened compounds of a structural similarity with retinoids and bearing a hydrophobic region and a terminal carboxyl group or its bioisostere, and excluded those acting through RARs. Through these screenings, we identified compounds 3 and 4. Compound 3 has a chemical formula of C₁₇H₁₆NO₃Cl, and compound 4 has a chemical formula of C₁₈H₁₇NO₃S (Fig. 1A). Compounds and atRA elicit
validate the functional role of these compounds in activating ERK1/2 through Crabp1, we performed an in vitro reaction. These data show that the holo-Crabp1 complex, with atRA, compound 3 or compound 4, can directly activate ERK1/2 activity. Mechanistically, this suggests that Crabp1 may act as a scaffold to regulate ERK activity, as shown in co-association of Crabp1 with immunoprecipitated ERK2 (Fig. 2B, lower panel). We then found that compound 3 like atRA, decreased cell viability in WT ESC but had no effect in Crabp1 KO ESC as detected in MTT assay (Fig. 2C). Further, like RA, these compounds also expanded the G1 phase in WT ESC (from 24.4% to 29.3 percent for RA and to 28.3 percent and 28.4 percent for compounds 3 and 4, respectively) but not in Crabp1 KO ESC (Fig. 2D). Interestingly, we found atRA rapidly elevated the level of protein phosphatase 2A (PP2A) activity that was abolished by silencing Crabp1 (Fig. 2E, left). ERK inhibition blocked elevation in PP2A activity (Fig. 2E, center), suggesting the non-genomic effect of atRA can be extended to PP2A that is important for cell cycle control. As an ultimate proof, we confirmed that atRA, and compounds 3 and 4 could no longer activate PP2A in Crabp1 KO ESCs (Fig. 2E, right).

Non-genomic ERK1/2 activation is lost in Crabp1-negative cancer cells. Several genetic association studies have reported correlation of reduced Crabp1 expression in certain cancers/tumors. As shown in Fig. 3A, Crabp1 expression was not detected in mouse ovarian cancer cell line (MOVCAR), glioma cell line GL261 or human embryonic kidney cell line 293T, but remained robust in Cos-1 and mouse ESC. However, MCF7 cancer cells do express CRABP1 (see later in Fig. 4D). Crabp1-negative cells may reflect either the lack of expression in certain cell populations that give rise to these cancer cells, or epigenetic changes of the original cells that give rise to these cancer cells. To test our hypothesis that Crabp1 is functionally involved in mediating ERK activation, we used MOVCCAR cell (Crabp1-negative) to examine the effect of Crabp1 in mediating rapid ERK1/2 activation by atRA and compounds 3 and 4. As shown in Fig. 3B, without expression of Crabp1 in MOVCCAR, atRA, compounds 3 and 4 failed to elicit early activation of ERK1/2. Crabp1 was then re-introduced into MOVCCAR cells and kinetics of ERK1/2 activation by atRA and compounds 3 and 4 were analyzed on western blots (Fig. 3C). The results show that compound 3 activates ERK1/2 at 0.5–2 hr and compound 4 transiently at 0.5–1 hr, suggesting non-genomic activation (upper panels). Similar to what we reported in ESC and Cos-1 cells, atRA biphassically activates ERK1/2 in MOVCCAR cells re-expressing Crabp1 (upper panel). This rapid activation by atRA and compounds 3 and 4 was not detected in cells without Crabp1 (lower panels). These data show that in a cancer cell context, Crabp1 expression is critical for rapid ERK1/2 activation by atRA and compounds 3 and 4.

Non-genomic ERK1/2 activation by compounds 3 and 4 is dependent on binding to Crabp1. Crabp1 is classically known for its role in binding and sequestering atRA in the cytoplasm to regulate intracellular atRA concentrations. Structural studies have provided insight into the interactions of atRA with the ligand-binding pocket of Crabp1. Based upon these structural data, we previously generated atRA-binding mutant Crabp1 specifically at residues arginine 131 and tyrosine 133. Arginine R131 to alanine (R131A) mutation severely abrogated Crabp1 interaction with atRA, but tyrosine 133 mutation, such as Y133F, did not. We thus used the R131A mutant Crabp1 to determine whether Crabp1’s ligand binding was required for activating ERK1/2. As shown in Fig. 3D, upon re-introducing R131A mutant Crabp1, atRA and compounds 3 and 4 remained unable to activate ERK1/2, whereas introducing the wild type Crabp1 successfully resumed ERK1/2 activation. As a control, the Y133F mutant Crabp1, which did not affect ligand binding, could still elicit non-genomic activation of ERK1/2. These data show that, ligand binding of Crabp1, which requires R131, is critical for the non-genomic action of atRA, as well as ERK activation by Crabp1 ligands. Finally, we directly confirmed the ligand binding ability of purified Crabp1 mutant proteins as shown in Fig. 3E.

Crabp1 ligands stimulate cancer cell apoptosis. The non-genomic activity of atRA/Crabp1 affects PP2A and modulates cell cycle progression in ESC (Fig. 2), which supports genetic association studies suggesting Crabp1 as a tumor suppressor. We thus monitored apoptosis in cancer cells with and without Crabp1 expression in pSIVA staining that detects surface phosphatidylserine. As shown in Fig. 4A, in Crabp1-negative MOVCCAR cells transfected with a control vector, neither atRA nor compounds 3 or 4 significantly changed pSIVA-positive cell populations. However, in MOVCCAR cells expressing wild type Crabp1, atRA, compounds 3 and 4 each triggered a robust increase in pSIVA-positive cell populations (approximately 60–70%, right panel). This is supported by key apoptosis marker Bcl-2 (Fig. 4B). Note that while the level of pro-apoptotic protein Bax was not altered, the antiapoptotic Bcl-2 level was reduced, resulting in a higher ratio of Bax over Bcl-2 expression. This further suggests that holo-Crabp1 elicited apoptosis may target (reduce) the effectiveness of anti-apoptotic
Figure 2. Crabp1-ERK1/2 activation by compounds 3 and 4 stimulates PP2A in ESC. (A) ERK activation elicited by RA and compounds 3 and 4 for 30 min is abolished in Crabp1 KO ESC. (B) Semi-in vitro kinase assay. Partially immuno-purified Crabp1 complex (bottom) activates recombinant ERK1/2 under 100 nM RA, C3, and C4 treatment for 30 min. (C) RA and Compound 3 decrease cell viability as detected by MTT assay with 100 nM treatments for 24 hrs. (D) G1 phase expansion by RA and compounds 3 and 4 in WT ESC but not in Crabp1 KO ESC. Cells were treated with RA and compounds 3 and 4 at 100 nM for 12 hrs before flow cytometric analysis. Asterisk shows significance: RA (p = 0.05), C3 (p = 0.05) and C4 (p = 0.04) versus control, and mean ± S.E.M (n = 4). (E) WT ESCs were transfected with scrambled (control) and siCrabp1 (Crabp1 KD) followed by RA treatment (100 nM). RA-induced phosphatase activity (detected for free phosphate) is blocked by Crabp1 knockdown. Asterisk shows significance: 30min (p = 0.01), 3hr (p = 0.002), and 6hr (p = 0.004) versus WT ESC (left). RA-stimulated PP2A activation is blocked by ERK1/2 inhibitor, FR180204; 3 hr (p = 0.03) versus vehicle (center). RA, compounds 3 and 4 stimulate PP2A activity after 100 nM treatment for 3 hrs in WT ESC but not in Crabp1 KO ESC. RA (p < 0.001), C3 (p = 0.017), and C4 (p = 0.001) versus control (right). Data (A–E) are representative of at least 3 independent experiments.
pathway. Very interestingly, mRNA levels of Bcl-2 were unaffected; suggesting holo-Crabp1 post-transcriptionally affects Bcl-2 protein level. Consistently, in the presence of ERK1/2 inhibitor FR180204 (Fig. 4C), atRA, compound 3 or compound 4 could no longer induce cleavage of caspase 3. Given the ability of atRA and compounds 3 and 4 to induce apoptosis in animal cancer cell line context, we surveyed several human cell lines and found that CRABP1 expression was lost in human ovarian cancer cell line A2780 and human pancreatic ductal carcinoma cell line KPC. Interestingly, in these CRABP1 negative cell lines, atRA, compounds 3 and 4 failed to activate ERK1/2 in 30 minutes (Fig. 4D, upper panels), or induce cleavage of caspase 3 after 24 hr treatment. This is in contrast to CRABP1-positive human breast cancer cell line MCF-7 in which apoptosis was readily induced by atRA and compounds 3 and 4, as indicated by the presence of cleaved caspase 3.

Discussion
In this study we determine the physiological role of Crabp1 in a stem cell context, which is to mediate atRA’s non-genomic signaling pathway in modulating cell cycle progression. We further identify specific ligands of

Figure 3. Crabp1-dependent activation of ERK1/2 by compounds 3 and 4 requires ligand binding. (A) Crabp1 expression assessed on protein (left) and mRNA (right) levels. (B) Western blot analysis. Compounds 3 and 4 fail to activate ERK1/2 in Crabp1-negative MOVCAR at 30 min, 100 nM. (C) Western blot analyses of kinetics of ERK1/2 activation by compounds (100 nM). MOVCAR cells re-expressing Crabp1 (upper panels) or empty vector (EV, lower panels) were treated with vehicle, RA, compound 3 or compound 4 for the indicated hrs. Early phase of ERK1/2 activation is detected in Crabp1 expressing cells. (D) Rapid ERK1/2 activation in MOVCAR re-expressing wild type or mock-mutated Crabp1 Y133F but no activation in cells re-expressing Crabp1 R131A, RA binding deficient mutant at 30 min, 100 nM treatments. (E) 3H-atRA ligand binding assay reveals RA binding to wild type and Crabp1 Y133F but no binding to Crabp1 R131A proteins. *p < 0.001 versus to control. Data is displayed as counts per minute (CPM). Data (A–E) are representative of at least 3 independent experiments.
Figure 4. Crabp1-dependent induction of apoptosis by compounds 3 and 4. (A) Images showing apoptosis of Crabp1-positive MOVCAr cells (pSIVA staining, green). Cells were treated with 100 nM for 24 hrs.
Quantitation of pSIVA positive cells shown on the right: *p < 0.001 versus control, n = 3, EV: empty vector.
(B) Decrease in Bcl-2 protein by atRA, compound 3 or 4 (left), without altering mRNA expression (right).
(C) Increase in cleaved caspase 3 (upper right), which is blocked by ERK1/2 inhibitor (below) in MOVCAr.
This is abolished in the absence of Crabp1 (upper left, EV) under 100 nM RA, C3, and C4 treatment for 24 hrs.
Quantification of cleaved caspase 3 is shown (upper, n = 4). (D) Compounds lose ability to induce ERK activity in human CRABP1 null cancer cell lines A2780 (ovarian) and KPC (pancreatic ductal carcinoma) after 100 nM, 30 min treatment (upper). Compounds are able to induce apoptosis after 24 hr treatment (lower) in CRABP1 positive MCF7 breast cancer cell line (lower right). Quantification of cleaved caspase 3 is shown for MCF-7. Data (A–D) are representative of at least 3 independent experiments.
Crabp1, which elicit activity mimicking the non-genomic activity of atRA without involving RARs. As a proof of concept, we examine these compounds, through binding to Crabp1, in enhancing Crabp1-positive cancer cell apoptosis. To this end, several genetic association studies have reported correlation of reduced Crabp1 expression in cancers/tumors. In serious ovarian adenocarcinomas and clear cell ovarian adenocarcinomas, reduced Crabp1 expression was tied to significantly poorer survival prognosis. Epigenetic studies in which promoter methylation status of several genes in primary ovarian carcinomas and their in vitro models were assayed showed that the Crabp1 gene promoter and the CpG islands of its coding region were hypermethylated – indicating a loss of Crabp1 expression. Our earlier studies have also reported that DNA methylation on the Crabp1 gene promoter is an important regulatory mechanism of its gene activity. In esophageal squamous-cell carcinoma cells, absence of Crabp1 promoted cell growth. Similar trends were found in colon cancer cell lines HCT116 and SW48 and in primary colorectal carcinomas, as well as in papillary thyroid carcinomas. This current study reveals a mechanism in which Crabp1 can play a role as a tumor suppressor, through non-canonical activation of ERK1/2 that then induces apoptotic pathways, likely through inhibiting anti-apoptosis, for cancer cell death. Importantly, these activities were abolished in Crabp1-negative cells. The identification of Crabp1 specific ligands, as well as its mechanism of action, sheds important insights into a potential new retinoid-based therapeutic strategy that can avoid toxicity associated with the genomic action mediated by RARs.

Multiple genes regulate the apoptotic pathway including Bcl-2 and Bax. Bcl-2 exerts anti-apoptotic effects through a variety of mechanisms including inhibition of caspase activation by blocking cytochrome C release from the mitochondria. Bcl-2 binds to Bax to prevent its ability to induce permeabilization of the mitochondrial membranes that trigger cytochrome C release and subsequent caspase activation. The ratio of these proteins is critical in determining the efficiency of apoptosis. A high level of Bax counteracts the anti-apoptotic effect of Bcl-2, and a high Bcl-2/Bax ratio is associated with various subtypes of cancer that are highly resistant to chemotherapies. Importantly, these activities were abolished in Crabp1-negative cells. The identification of Crabp1 specific ligands, as well as its mechanism of action, sheds important insights into a potential new retinoid-based therapeutic strategy that can avoid toxicity associated with the genomic action mediated by RARs.

Further development of Crabp1-selective ligands may prove efficacious in therapeutic application of retinoids. Compounds 3 and 4 elicit strong pro-apoptotic activity through binding to Crabp1. It is interesting to note that while these compounds do not bind to Crabp1 as tightly as atRA, they are still able to induce, almost as efficiently as atRA, non-canonical activation of ERK1/2. The fact that these compounds failed to elicit activities through mutant Crabp1 defective in atRA binding would suggest that occupying this ligand binding pocket is important for the activation of Crabp1 to elicit ERK activity. Structural studies may yield insight into how these molecules directly bind to Crabp1 and provide a basis for more rational drug design in the future.

Materials and Methods

Compounds. The Kagechika chemical compound library includes about 5,000 compounds that were synthesized in his laboratory for different purposes, including targeting nuclear receptors, in TMDU. Fifty compounds were selected from this library by the structural similarity with known retinoids, bearing some hydrophobic region and the terminal carboxyl group or its bioisostere. The structures of hit compounds 3 and 4 were confirmed by high resolution mass spectrometry. Compounds 3 and 4 are synthesized in his laboratory for different purposes, including targeting nuclear receptors, in TMDU. Fifty compounds were selected from this library by the structural similarity with known retinoids, bearing some hydrophobic region and the terminal carboxyl group or its bioisostere. The structures of hit compounds 3 and 4 were confirmed by high resolution mass spectrometry. Compounds 3 and 4 are confirmed by high resolution mass spectrometry. Compounds 3 is known as 3-(2-(4-Chloro-N-methylbenzamido) phenyl)propanoic acid (37) and compound 4 as (E)-3-(2-(N-Methyl-4-(methylthio) benzamido) phenyl)propanoic acid.

Cell culture methods, plasmids. Cos-1 cells, ESC and MOV CAR were maintained as described in medium containing dextran-coated charcoal-treated fetal bovine serum. MOV CAR, A2780, KPC cell lines were gifts from Dr. Sundaram Ramikrishnan, University of Minnesota. Plasmid transfection was conducted using Lipofectamine 3000 (Invitrogen). Mouse Crabp1 cDNA was cloned into pCMX-PL1. Mutations of Crabp1 R131A, and T133F were introduced using site directed mutagenesis (Stratagene).

ESC generation. Crabp1 knockout ESC were established previously, and used to establish Crabp1 knockout mice. Crabp1 KO ESC were derived from day 2.5 morula from Crabp1 KO mice as described. All animal procedures have been approved and were performed according to the University of Minnesota IACUC Animal Care Committee guidelines.

Western blotting and chemicals. Whole cell lysate was prepared as described. Antibodies for β-actin (SC-47778), ERK1 (SC-93), and ERK2 (SC-153) were from Santa Cruz. Antibodies for Crabp1 (C1608), Igp (F3165) were from Sigma. Anti-phospho-ERK1/2 (9101) and cleaved caspase-3 (9661) were from Cell Signaling. Anti-GST (05–311) was from Upstate. atRA (100 nM) was from Sigma. AGN 193109 (RAR antagonist, 100 nM) was from Santa Cruz. 5-(2-phenyl-pyrazolo[1,5-a] pyridin-3-yl)-1H-pyrazolo[3,4-c]pyridazin-3-ylamine (ERK1/2 inhibitor, 1 μM) was from EMD. 3H-RA was from Perkin Elmer.

In vitro ligand binding competition assay. In vitro ligand binding competition assay was as described. Recombinant His-Crabp1 was purified from bacteria. In 300 μl of binding buffer (50 mM HEPES, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40), an equimolar concentration of Crabp1 (100 nM) and [3H]-atRA was incubated for 40 min at room temperature in the presence of excess cold ligand (1 μM). His-tagged Crabp1 was affinity-captured on nickel-nitrilotriacetic acid-agarose affinity beads (Qiagen) for 2 hrs at 4°C, and washed twice with binding buffer. Ligand-bound Crabp1 was dispersed in scintillation mixtures, and the radioactivity was measured in a liquid scintillation counter (Beckman).
**In vitro ligand binding assay.** His-Crabp1 (WT, R131A, and Y133F) proteins were purified from bacteria. In 300 μl of binding buffer (50 mM HEPES, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 0.1% Nonidet P-40, pH 8.0), an equimolecular concentration of Crabp1 and [3H]RA was incubated for 40 min at room temperature. His-tagged Crabp1 was affinity-captured on nickel–nitrotriacetic acid–agarose affinity beads (Qiagen) for 2 hrs at 4°C, and washed twice with binding buffer. Ligand-bound Crabp1 was dispersed in scintillation mixtures, and the radioactivity was measured in a liquid scintillation counter (Beckman).

**In vitro kinase assay.** Semi in-vitro kinase assay was performed as described10. Cos-1 cells expressing flag-Crabp1 were lysed and immunoprecipitated with anti-flag antibody. The precipitated complex was incubated with pure 0.4 μg GST-ERK2 (14–198, Upstate) in 40 μl kinase buffer (20 mM MOPS pH 7.2, 25 mM β-glycerolphosphate, 5 mM EGTA, 1 mM Sodium orthovanadate, 1 mM DTT, 120 μM ATP, 18 mM MgCl₂, 1X Protease inhibitor) at 30°C for 30 min. Samples separated on SDS-PAGE gel were detected with atRA and C3 treatment.

**Phosphatase assay.** Serine/threonine phosphatase (V2460, Promega) and PP2A phosphatase (17–313, Upstate) assays were performed as manufacturer’s instructions. Reaction times were 10 min at 30°C. ESC were treated with 100 nM atRA, C3, or C4 for indicated time points. Phosphatase activity was detected at optical density 630 nm in a microplate reader (Tecan M1000).

**Flow cytometry.** After treatment of 100 nM for 12 hrs, cells were harvested and fixed with 70% ethanol overnight at −20°C. Cells were then stained with staining buffer (1XPBS containing 100 μg/ml DNase-free RNase A and 40 μg/ml propidium iodide) at 37°C for 30 min. Cells were analyzed with fluorescence-activated cell sorting (BD Accuri).

**MTT assay.** Cell viability was assessed by measuring their ability to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) using the TOX-1 kit (Sigma Aldrich) at 24 hr timepoint after 100 nM atRA and C3 treatment.

**Detection of apoptosis.** Cell apoptosis was detected using CytoGLO pSIVA IANBD kit (IMG-6701K, IMGENEX) Images were acquired by Olympus FluoView 1000 IX2 upright confocal microscope. The fluorescence intensity representing pSIVA positive cell number from different fields was counted using ImageJ and quantified.

**Data analysis.** Analyses of data were performed using appropriate analysis of variance. Significant effects were followed with appropriate post hoc tests. In all cases, statistic analyses were done by two-tailed Student’s t-test, and the comparison was considered statistically significant when p < 0.05. Data were presented as means ± S.E.M. All experiments used an N of three unless otherwise indicated.

**References**

1. Marino, M., Galluzzo, P. & Ascenzi, P. Estrogen signaling multiple pathways to impact gene transcription. *Curr Genomics* 7, 497–508 (2006).
2. Simoncini, T. *et al.* Genomic and non-genomic effects of estrogens on endothelial cells. *Steroids* 69, 537–542 doi: 10.1016/j.steroids.2004.05.009 (2004).
3. Jordan, V. C. The new biology of estrogen-induced apoptosis applied to treat and prevent breast cancer. *Endocr Relat Cancer* 22, R1–31, doi: 10.1530/ERC-14–0448 (2015).
4. Kalyanaraman, H. *et al.* Nongenomic thyroid hormone signaling occurs through a plasma membrane-localized receptor. *Sci Signal* 7, ra48, doi: 10.1126/scisignal.2004911 (2014).
5. Mitre-Aguilar, I. B., Cabrera-Quintero, A. J. & Zentella-Dehesa, A. Genomic and non-genomic effects of glucocorticoids: implications for breast cancer. *International journal of clinical and experimental pathology* 8, 1–10 (2015).
6. Kalyanaraman, H. *et al.* Overlapping nongenomic and genomic actions of thyroid hormone and steroids. *Best practice & research. Clinical endocrinology & metabolism* 27, 581–593, doi: 10.1016/j.beem.2015.04.001 (2015).
7. Al Tanoury, Z., Piskunov, A. & Rochette-Egly, C. Vitamin A and retinoid signaling: genomic and nongenomic effects. *J Lipid Res* 54, 1761–1775, doi: 10.1194/jlr.R030833 (2013).
8. Ochoa, W. E. *et al.* Retinoic acid binds to the C2-domain of protein kinase C(alpha). *Biochemistry* 42, 8774–8779, doi: 10.1021/bi034713g (2003).
9. Chen, N. & Napoli, J. L. All-trans-retinoic acid stimulates translation and induces spine formation in hippocampal neurons through a membrane-associated RARalpha. *J. Cell Sci.* 22, 236–245, doi: 10.1096/jcs.07–8739com (2008).
10. Persaud, S. D., Lin, Y. W., Wu, C. Y., Kagechika, H. & Wei, L. N. Cellular retinoic acid binding protein I mediates rapid non-canonical activation of ERK1/2 by all-trans retinoic acid. *Cell Signal* 25, 19–25, doi: 10.1016/j.cellsig.2012.09.002 (2013).
11. Gupta, P. *et al.* Retinoic acid-stimulated sequential phosphorylation, PML recruitment, and SUMOylation of nuclear receptor TR2 to suppress Oct4 expression. *Proc Natl Acad Sci USA* 105, 11424–11429, doi: 10.1073/pnas.0710561105 (2008).
12. Yoon, B. S. *et al.* Optimal suppression of protein phosphatase 2A activity is critical for maintenance of human embryonic stem cell self-renewal. *Stem Cells* 28, 874–884, doi: 10.1002/stem.412 (2010).
13. Lo-Coco, F. *et al.* Retinoic acid and arsenic trioxide for acute promyelocytic leukemia. *N Engl J Med* 359, 111–121, doi: 10.1056/NEJMoa1308074 (2013).
14. Niles, R. M. Biomarker and animal models for assessment of retinoid efficacy in cancer chemoprevention. *Acta Pharmacol Sin* 28, 1383–1391, doi: 10.1111/j.1745–7254.2007.00685.x (2007).
15. Wu, K. *et al.* Suppression of mammary tumorigenesis in transgenic mice by the RXR-selective retinoid, LGD1069. *Cancer Epidemiol Biomarkers Prev* 11, 467–474 (2002).
16. McCormick, D. L. & Rao, K. V. Chemoprevention of hormone-dependent prostate cancer in the Wistar-Unilever rat. *Eur Urol* 35, 464–467, doi: 10.1016/S0181–7229 (1999).
17. Thompson, H. J., Meeker, L. D. & Becci, P. J. Effect of combined selenium and retinyl acetate treatment on mammary carcinogenesis. *Cancer Res* 41, 1413–1416 (1981).
18. Becci, P. J.* et al.* N-butyl-N-(4-hydroxybutyl)nitrosamine-induced urinary bladder cancer in C57BL/6 X DBA/2 F1 mice as a useful model for study of chemoprevention of cancer with retinoids. *Cancer Res* 41, 927–932 (1981).
24. Bushue, N. & Wan, Y. J. Retinoid pathway and cancer therapeutics.

23. Merino, D., Lok, S. W., Visvader, J. E. & Lindeman, G. J. Targeting BCL-2 to enhance vulnerability to therapy in estrogen receptor-positive breast cancer. Oncogene, doi: 10.1038/onc.2015.287 (2015).

22. Tang, X. H. & Gudas, L. J. Retinoids, retinoic acid receptors, and cancer. Annual review of pathology 6, 345–364, doi: 10.1146/annurev-pathol-011110-130303 (2011).

21. Merino, D., Lok, S. W., Visvader, J. E. & Lindeman, G. J. Targeting BCL-2 to enhance vulnerability to therapy in estrogen receptor-positive breast cancer. Oncogene, doi: 10.1038/onc.2015.287 (2015).

20. Clarke, J. M., Huo, Y. G., Ariga, K. & Lennard, P. W. L. Retinoids: potential in cancer prevention and therapy. Expert Rev Mol Med 6, 1–23, doi: 10.1017/S1462399404008488 (2004).

19. Curphey, T. J., Kuhlmann, E. T., Roebuck, B. D. & Longnecker, D. S. Inhibition of pancreatic and liver carcinogenesis in rats by retinoid- and selenium-supplemented diets. Pancreas 3, 36–40 (1988).

18. di Masi, A. et al. Retinoic acid receptors: from molecular mechanisms to cancer therapy. Mol Aspects Med 41, 1–115, doi: 10.1016/j.mam.2014.12.003 (2015).

17. Tang, X. H. & Gudas, L. J. Retinoids, retinoic acid receptors, and cancer. Annual review of pathology 6, 345–364, doi: 10.1146/annurev-pathol-011110-130303 (2011).
Author Contributions
S.D.P., S.-W.P. and L.-N.W. designed research; S.D.P., S.-W.P., M.I.-Y. and N.K.-N. performed research; H.K. and L.-N.W. contributed new reagents/tools; S.D.P., S.-W.P. and L.-N.W. analyzed data; and S.D.P., S.-W.P. and L.-N.W. wrote the paper.

Additional Information
Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Persaud, S. D. et al. All trans-retinoic acid analogs promote cancer cell apoptosis through non-genomic Crabp1 mediating ERK1/2 phosphorylation. Sci. Rep. 6, 22396; doi: 10.1038/srep22396 (2016).

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/
**Corrigendum:** All trans-retinoic acid analogs promote cancer cell apoptosis through non-genomic Crabp1 mediating ERK1/2 phosphorylation

Shawna D. Persaud, Sung Wook Park, Mari Ishigami-Yuasa, Naoko Koyano-Nakagawa, Hiroyuki Kagechika & Li-Na Wei

*Scientific Reports* 6:22396; doi: 10.1038/srep22396; published online 03 March 2016; updated 20 July 2016

This Article contains errors. In the last paragraph of the Results section,

"Given the ability of atRA and compounds 3 and 4 to induce apoptosis in animal cancer cell line context, we surveyed several human cell lines and found that CRABP1 expression was lost in human ovarian cancer cell line A2780 and human pancreatic ductal carcinoma cell line KPC.

should read:

"Given the ability of atRA and compounds 3 and 4 to induce apoptosis in animal cancer cell line context, we surveyed several additional cell lines and found that CRABP1 expression was lost in human ovarian cancer cell line A2780 and mouse pancreatic ductal carcinoma cell line KPC.

In addition, the legend of Figure 4D,

"(D) Compounds lose ability to induce ERK activity in human CRABP1 null cancer cell lines A2780 (ovarian) and KPC (pancreatic ductal carcinoma) after 100 nM, 30 min treatment (upper). Compounds are able to induce apoptosis after 24 hr treatment (lower) in CRABP1 positive MCF7 breast cancer cell line (lower right). Quantification of cleaved caspase 3 is shown for MCF-7. Data (A–D) are representative of at least 3 independent experiments.

should read:

"(D) Compounds lose ability to induce ERK activity in human CRABP1 null cancer cell line A2780 (ovarian) and mouse KPC (pancreatic ductal carcinoma) after 100 nM, 30 min treatment (upper). Compounds are able to induce apoptosis after 24 hr treatment (lower) in CRABP1 positive MCF7 breast cancer cell line (lower right). Quantification of cleaved caspase 3 is shown for MCF-7. Data (A–D) are representative of at least 3 independent experiments.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/