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14. ABSTRACT

Radiation therapy is a first line treatment for prostate cancer patients and the patient's response is generally good. However, approximately 10% of low grade and up to 60% of high grade of patients will experience tumor recurrence. We have found that following a clinical radiation therapy protocol (2 Gy/day, 5 days/week), ionizing radiation (IR) induced the LNCaP prostate cancer cells to transdifferentiate into neuroendocrine-like (NE-like) cells, a process also known as neuroendocrine differentiation (NED) that is associated with disease progression and the acquisition of androgen-independent growth. Once differentiated, the NE-like cells are highly resistant to radiation and survive the treatment without any obvious cell death. Furthermore, we have demonstrated that two transcription factors, CREB and ATF2 that can bind the same DNA sequence for gene transcription, are involved in this process. Irradiated cells accumulate a large amount of ATF2 in the cytoplasm concurrent with an increased amount of phosphorylated form of CREB (pCREB) in the nucleus. Further evidence suggests that ATF2 acts as a transcriptional repressor and CREB functions as a transcriptional activator in NED. Significantly, we also found that IR-induced NE-like cells are reversible and dedifferentiated cells are cross-resistant to radiation, androgen ablation and chemotherapeutic agent docetaxel treatments. These findings suggest that radiation-induced NED may represent a novel pathway by which prostate cancer cells survive the treatment and contribute to recurrence.

15. SUBJECT TERMS
LNCaP, prostate cancer, ionizing radiation, neuroendocrine differentiation, ATF2, CREB

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Introduction

The neuroendocrine (NE) cells are one of the three types of epithelial cells in the human prostate, and are present in almost all cases of prostatic adenocarcinoma [1, 2]. Although the physiological role of NE cells in the normal prostate remains unclear, increased number of NE-like cells is found in advanced prostate cancer. Accumulated evidence suggests that NE-like cells produce a number of growth factors or peptide hormones that facilitate the growth of surrounding tumor cells in a paracrine manner, and that NE-like cells are clinically associated with androgen-independent growth of prostate cancer [1-4]. Consistent with this, androgen ablation can also induce NE differentiation (NED) of prostate cancer *in vitro* and *in vivo* [4-10]. Hence, the number of NE-like cells appears to be an indicator of prostate cancer progression. In addition to androgen ablation, IL-6 [11-16] and agents that can elevate the intracellular levels of cAMP [12, 17-20] also induce NED. Recently, our preliminary results showed that ionizing radiation (IR) also induces NED *in vitro*. Interestingly, we observed that irradiated cells showed increased cytoplasmic localization of activating transcription factor 2 (ATF2) and an increase in the phosphorylated form of cAMP response element binding (CREB) protein. Since ATF2 and CREB both belong to the basic region leucine zipper (bZIP) family of transcription factors and bind the same cAMP response element (CRE) as a homodimer or a heterodimer to regulate gene transcription [21]. While some target genes can be activated by CREB and ATF2 equally or cooperatively [22-24], differential regulation of other target genes by CREB and ATF2 has also been observed [25-29]. Importantly, CREB is implicated in prostate cancer growth [30], acquisition of androgen independent growth [31], cAMP-induced NED [18, 32], and transcription of prostate-specific antigen [33]. Based on these preliminary findings, we proposed that ATF2 may function as a transcriptional repressor and CREB acts as a transcription activator of NED. Hence, IR induces NED by sequestering ATF2 in the cytoplasm and activating CREB in the nucleus. To determine how ATF2 and CREB NED at the transcriptional level, we proposed three specific aims in the applications: **(1)** determine functional relationship between CREB and ATF2 in IR-induced NED; (**2**) elucidate molecular mechanisms underlying regulation of IR-induced NED by pCREB and ATF2; **(3)** identify cytoplasmic signals responsible for the cytoplasmic sequestration of ATF2 and nuclear accumulation of pCREB. Given that NE-like cell is an indicator of cancer progression and that NE-like cells are highly resistant to radiation- and other agents-induced apoptosis, our novel observation that IR can induce NED of prostate cancer cells uncovers a novel pathway by which tumor cells may develop radioresistance. Successful completion of this project will allow for the development of novel radiosensitization approaches by targeting NED at the transcriptional regulation level.

Body

Completion of Approved Statement of Work

Task 1. Aim 1: Determine the functional relationship between CREB and ATF2 in IR-induced NED (Months 1-18)

a. We will first use LNCaP cells to establish stable cell lines expressing tetracycline repressor (Months 1-3)
b. Subclone our current two siRNA constructs for both CREB and ATF2 into pRNATin-H1.2/Neo vector and stably transfect them into repressor-expressing cell lines to isolate stably integrated clones (Months 2-6).
c. Subclone cDNAs encoding different CREB and ATF2 mutants into pcDNA4/TO vector (Months 4-6).
d. Establish stable cell lines expressing siRNA and different mutant CREB and ATF2 proteins (Months 6-12).
e. Optimize expression conditions (Months 7-10).
f. Examine knockdown effect of CREB and ATF2 on IR-induced NED (Months 7-18).
g. Examine the effect of overexpressed constitutively cytoplasmic- and nuclear-localized pCREB or ATF2 on IR-induced NED (Months 7-18).

We have successfully used LNCaP cells to establish several stable cell lines expressing tetracycline repressor. Screening of these stable cell lines identified one that showed high expression of the tetracycline repressor. This cell line was also used to establish several stable cell lines [pcDNA4/TO, pcDNA4/TO-CREB(S133A), pcDNA4/TO-nATF2, pRNATin-H1.2/Neo-ATF2shRNA, pRNATin-H1.2/Neo-CREBshRBA)]. Three stable cell lines expressing the dominant negative mutant CREB (CREB-S133A) and the constitutively nuclear-localized ATF2 (nATF2) were used to assess their role in IR-induced NED. We found that induction of CREB-S133A or nATF2 inhibited IR-induced NED. The results have been presented in our Cancer Research paper [34]. Results are presented in Figure 4 (page 9667) and methods are presented on page 9664.

We have also demonstrated that overexpression of a constitutively cytoplasmic-localized ATF2 induced NED. This is due to the sequestration of endogenous ATF2 in the cytoplasm by cATF2. This result is similar to NED induced by ATF2 knockdown in transient transfection. These results are presented in Figure 3 (page 9666) and Supplemental Figures 2 and 3.

In summary, we have completed all the proposed experiments in Aim 1 except that induction of shRNA in several stable cell lines did not knockdown ATF2 and CREB (Task f), despite the fact that the same sequences used in pSUPER vector for transient tranfection worked pretty well. We are continuing to screen for new clones and we are also planning to purchase new plasmid vectors for establishing inducible and stable cell lines to knockdown CREB and ATF2. Once this task is completed, we will test the effect of knocking down CREB and ATF2 on IR-induced NED.

Task 2. Aim 2: Elucidate molecular mechanisms underlying the promotion of NED by pCREB and the repression by ATF2 (Months 12-24)

We have not completed any of tasks in this Aim 2. We are getting start to work on this aim.

Task 3. Aim 3: Determine how IR induces cytoplasmic sequestration of ATF2 and identify cytoplasmic signals that may regulate subcellular localization of pCREB and ATF2 (Months 12-36)

We have not completed any of tasks in this Aim 2. We are getting start to work on this aim.

Additional accomplishments beyond the Approved SOW

We have also demonstrated that IR-induced NED is reversible. Importantly, we have isolated three dedifferentiated clones and found that these dedifferentiated cells have acquired the ability to be cross-resistant to radiation, androgen ablation, and chemotherapeutic agent docetaxel. Also, these dedifferentiated clones respond poorly to IR- or androgen ablation-induced NED. These findings strongly suggest that IR-induced NED may represent a novel pathway by which prostate cancer cells survive the treatment and contribute to recurrence. These results are presented in Figures 5 and 6 (page 9668) and Supplementary Figures 4 and 5 in the Cancer Research paper [34].

Key Research Accomplishments

- Demonstrated that 40 Gy of irradiation is sufficient to induce neurite extension in LNCaP cells
- Demonstrated that 40 Gy of irradiation can induce expression of two NE markers chromagranin A (CgA) and neuron specific enolase (NSE)
- Demonstrated that IR-induced NE-like cells show increased cytoplasmic localization of ATF2 and increased pCREB in the nucleus
- Demonstrated that 10 Gy of irradiation is enough to induce cytoplasmic sequestration of ATF2 and nuclear accumulation of pCREB
- Demonstrated that knockdown of ATF2 or overexpression of a constitutively cytoplasmic-localized ATF2 induces NED
- Demonstrated that overexpression of VP16-bCREB induces NED and its induction of NED can be attenuated by a constitutively nuclear-localized ATF2 (nATF2)
- Demonstrated that overexpression of nATF2 or CREB-S133A (dominant negative mutant of CREB) can inhibit IR-induced neurite outgrowth. However, only nATF2, but not CREB-S133A, inhibits IR-induced CgA and NSE expression.
- Demonstrated that IR-induced NED is reversible and dedifferentiated cells are cross-resistant to the treatments with radiation, androgen ablation and chemotherapeutic agent docetaxel

Reportable Outcomes

1. Publication of research results in Cancer Research
   Deng, X., Liu, H., Huang, J., Cheng, L., Keller, E, Parsons, S.J. and Hu, C.D. Ionizing radiation induces prostate cancer cell neuroendocrine differentiation through interplay of CREB and ATF2: Implications for disease progression. *Cancer Res.* **68**:9663-9670 (2008)
2. Meeting attendance
   The interplay of CREB and ATF2 in regulating ionizing radiation-induced neuroendocrine differentiation in prostate cancer cells
   Authors: Xuehong Deng[1], Han Liu[1], Jiaoti Huang[2], Liang Cheng[3], Evan T. Keller[4], Sarah J. Parsons[5], and Chang-Deng Hu[1]
   Meeting: Mechanisms and Models of Cancer
   Place and Date: Cold Spring Harbor, August 13-17, 2008
3. Invited Seminars
   (1) Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy
   Place: University of Virginia Cancer Center
   Date: December 18, 2008
   (2) Ionizing radiation-induced neuroendocrine differentiation: implication in prostate cancer therapy
   Place: Indiana University Medical School, Department of Bioehcmistry
   Date: February 2, 2009
4. Development of cell lines
   We have isolated three radiation resistant clones LNCaP-IRR1, LNCaP-IRR2 and LNCaP-IRR3 from dedifferentiated cells. These clones will be useful for molecular mechanism study and for development of novel therapeutics.
5. Funding applied and funded
   Title: Targeting of prostate cancer transdifferentiation and proliferation via a novel DNA nanotube-based nucleic acid delivery
   Agency: Lilly Seed Grant (School of Pharmacy and Pharmaceutical Sciences, Purdue)
   Total Cost: $100,000
   Period: Jan 2009 – Dec 2010

Conclusion

Under the support of this prostate cancer idea development award, we have demonstrated that ionizing radiation can induce neuroendocrine differentiation (NED) in the prostate cancer cells LNCaP. Furthermore, we have shown that two CRE-binding transcription factors ATF2 and CREB plays an opposite role in neuroendocrine differentiation and that IR induces NED by impairing ATF2 nuclear import and promoting nuclear localization of phosphorylated CREB. Importantly, we have also shown that IR-induced NED is reversible and three radiation resistant clones derived from dedifferentiated cells are cross-resistant to radiation, androgen ablation and chemotherapy. These findings suggest that IR-induced NED may represent a novel pathway by which prostate cancer cells survive the treatment and contribute to recurrence. Continuation of the proposed experiments in Aim 2 and 3 will define the molecular mechanism by which CREB and ATF2 oppose each other and identify upstream signaling that regulates subcellular localization and activation of ATF2 and CREB.

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THE INTERPLAY OF CREB AND ATF2 IN REGULATING IONIZING RADIATION-INDUCED NEUROENDOCRINE DIFFERENTIATION IN PROSTATE CANCER CELLS

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Radiation therapy is a first line treatment for prostate cancer patients with localized tumors. Although most patients respond well to the treatment, approximately 10-60% of prostate cancer patients experience recurrent tumors. However, the molecular mechanisms underlying tumor recurrence remain largely unknown. Here we show that ionizing radiation (IR) induces transdifferentiation of LNCaP prostate cancer cells into neuroendocrine (NE)-like cells, which are known to be implicated in prostate cancer progression, androgen independent growth and poor prognosis. Further analyses reveal that two CRE-binding transcription factors CREB and ATF2 play opposing roles in NE-like transdifferentiation, and that IR induces NE-like transdifferentiation by increasing nuclear content of phospho-CREB and impairing nuclear import of ATF2, a favorable ratio of pCREB over ATF2 in the nucleus for neuroendocrine differentiation. The IR-induced NE-like cells are reversible, and three IR-resistant clones isolated from dedifferentiated cells have acquired the ability to proliferate, but respond poorly to IR- and androgen depletion treatment in terms of NE-like redifferentiation. Significantly, these clones are cross-resistant to IR, chemotherapy, and androgen depletion treatments. These results suggest that radiotherapy-induced NE-like transdifferentiation represents a novel pathway by which prostate cancer cells survive the treatment and contribute to tumor recurrence.

Ionizing Radiation Induces Prostate Cancer Neuroendocrine Differentiation through Interplay of CREB and ATF2: Implications for Disease Progression

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Abstract

Radiation therapy is a first-line treatment for prostate cancer patients with localized tumors. Although some patients respond well to the treatment, ~10% of low-risk and up to 60% of high-risk prostate cancer patients experience recurrent tumors. However, the molecular mechanisms underlying tumor recurrence remain largely unknown. Here we show that fractionated ionizing radiation (IR) induces differentiation of LNCaP prostate cancer cells into neuroendocrine (NE)-like cells, which are known to be implicated in prostate cancer progression, androgen-independent growth, and poor prognosis. Further analyses revealed that two cyclic AMP–responsive element binding transcription factors, cyclic AMP–response element binding protein (CREB) and activating transcription factor 2 (ATF2), function as a transcriptional activator and a repressor, respectively, of NE-like differentiation and that IR induces NE-like differentiation by increasing the nuclear content of phospho-CREB and cytoplasmic accumulation of ATF2. Consistent with this notion, stable expression of a non-phosphorylatable CREB or a constitutively nuclear-localized ATF2 in LNCaP cells inhibits IR-induced NE-like differentiation. IR-induced NE-like morphologies are reversible, and three IR-resistant clones isolated from dedifferentiated cells have acquired the ability to proliferate and lost the NE-like cell properties. In addition, these three IR-resistant clones exhibit differential responses to IR- and androgen depletion–induced NE-like differentiation. However, they are all resistant to cell death induced by IR and the chemotherapeutic agent docetaxel and to androgen depletion–induced growth inhibition. These results suggest that radiation therapy–induced NE-like differentiation may represent a novel pathway by which prostate cancer cells survive the treatment and contribute to tumor recurrence. [Cancer Res 2008;68(23):9663–70]

Introduction

Radiation therapy is a first-line treatment for prostate cancer. Although some patients with localized tumors respond well to the treatment (1), ~10% of low-risk and up to 60% of high-risk prostate cancer patients experience recurrent tumors (2). However, the molecular mechanisms underlying tumor recurrence remain largely unknown.

Neuroendocrine (NE) cells are one of three types of epithelial cells in the human prostate and are present in 30% to 100% cases of prostatic adenocarcinoma (3, 4). Although the physiologic role of NE cells remains unclear, increased numbers of NE-like cells seem to be associated with prostate cancer progression, androgen-independent growth, and poor prognosis (5, 6). Interestingly, androgen ablation, cytokines such as interleukin 6 (IL-6), and agents that elevate the intracellular levels of cyclic AMP (cAMP) can induce NE-like differentiation (NED) in LNCaP prostate cancer cells by activating several distinct signaling pathways (5, 6). Like NE cells, the differentiated NE-like cells also produce a number of neuropeptides that facilitate the growth of surrounding tumor cells in a paracrine manner (5–7). They are generally androgen receptor negative (8, 9), highly resistant to apoptosis (10, 11), and their differentiation state is reversible (12). Thus, NE-like cells may survive in a dormant state and contribute to prostate cancer recurrence on dedifferentiation (12).

cAMP response element binding protein (CREB) belongs to the basic region leucine zipper (bZIP) family of transcription factors (13–15). It functions as a homodimer or heterodimer to bind a specific DNA sequence, the cAMP responsive element (16), to regulate transcription of target genes responsible for many cellular processes including cell proliferation and differentiation (15). CREB is implicated in prostate cancer growth (17), acquisition of androgen-independent growth (18), and transcription of chromogranin A (CgA; ref. 19) and prostate-specific antigen (20). Although it is known that CREB is activated by protein kinase A through the phosphorylation at Ser^{133} of CREB1B in response to cAMP (14, 21), whether CREB itself can induce NED remains to be determined.

Activating transcription factor 2 (ATF2) also belongs to the bZIP family of transcription factors (22, 23) and is a member of the activator protein 1 (AP-1; ref. 24). AP-1 activity is required for many cellular processes, and deregulated AP-1 activity is implicated in many cancers including prostate cancer (25). Interestingly, ATF2 and CREB share the same cAMP responsive element sequence and regulate the transcription of cAMP responsive element–containing genes. Whereas some cAMP responsive element–containing target

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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genes are activated by CREB and ATF2 equally or cooperatively (26), differential regulation of other target genes by CREB and ATF2 has also been observed (27–31). Unlike CREB, the role of ATF2 in prostate cancer is little known. A recent study reported that increased cytoplasmic localization of phospho-ATF2 in prostate cancer specimens correlates with the clinical progression of prostate cancer (32), suggesting that alteration of ATF2 subcellular localization may contribute to clinical progression of prostate cancer.

We recently showed that ATF2 is a nucleocytoplasmic shuttling protein and its subcellular localization is regulated by AP-1 dimerization (33). Here we present evidence that ATF2 constantly shuttles between the cytoplasm and nucleus in proliferating LNCaP cells and that fractionated ionizing radiation (IR) induces NED by impairing the nuclear import of ATF2 and increasing the nuclear phospho-CREB at Ser^{133} (pCREB).

Materials and Methods

Plasmid construction. To construct a constitutively activated form of CREB, cDNA encoding residues 413 to 490 of VP16 was amplified by PCR from VP16 (Clontech) and subcloned into pHA-CMV. To make VP16-bCREB fusion proteins, cDNA encoding the bZIP domain of CREB1B (residues 285–314) was amplified by PCR from a human cDNA library and subcloned into pHA-VP16. A flexible glycine spacer ($GGGGSx_4$) was inserted between VP16 and bCREB. For the construction of nuclear-localized ATF2 (nATF2), the sequence encoding a nuclear localization signal (PKKKRKV) from the large T antigen of SV40 (34) was subcloned upstream of ATF2 coding sequences in pFlag-ATF2. pFlag-cATF2 is a deletion mutant of ATF2 in which two nuclear localization signals are deleted (33). Both cytoplasmic-localized ATF2 (cATF2) and nATF2 were expressed as a fusion protein with the fluorescent protein, Venus, in transient transfection experiments. To knock down ATF2, sense and antisense oligos (19-mer) were synthesized and subcloned into pSUPER (OligoEngine). Four short interference RNA (siRNA) constructs were made, and their effect on ATF2 expression in LNCaP cells was verified by transient transfection, followed by immunoblotting of ATF2. One ATF2 siRNA construct targeting the 5′ untranslated region (148–167 of ATF2 mRNA) proved to be the most potent and was used in this work. All plasmids were verified by DNA sequencing.

IR-induced NE-like differentiation. Cells were cultured in 10-cm dishes in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics and were continuously irradiated (2 Gy/d, 5 d/wk) in a GC-220 Co-60 for the indicated times. NE-like cells were visualized by morphologic changes, and the induction of the NE markers, CgA and neuron-specific enolase (NSE), was determined by immunoblotting with anti-CgA and anti-NSE antibodies (Abcam). To determine the effect of CREB-S133A and nATF2 on IR-induced NED, we used a tetracycline-on system (Invitrogen) to establish stable cell lines that inducibly expressed HA-CREB-S133A or Flag-nATF2. The established cell lines were maintained in the presence of selectable markers (zeocin and blasticidin), and 5 μg/mL tetracycline was applied while cells were irradiated as described above. Media were changed twice a week, and antibiotics and tetracycline were added accordingly. Cells that extended neurites longer than two cell bodies were scored as differentiated, and the induction of CgA and NSE was analyzed by immunoblotting and quantified using ImageJ software. Values were normalized to β-actin.

Analysis of ATF2 and CREB subcellular localization. LNCaP cells were fixed in ice-cold 3.7% formaldehyde for 20 min, followed by permeabilization in ice-cold 0.2% Triton X-100 for 5 min. Cells were incubated with anti-ATF2 (c-19; Santa Cruz Biotechnology) overnight, followed by three washes and incubation with the secondary antibody conjugated with Texas red (Jackson ImmunoResearch Laboratories) for 1 h. To stain DNA, 4′,6-diamidino-2-phenylindole (DAPI) was added to the secondary antibody staining reaction at the final concentration of 0.5 μg/mL. Subcelluar localization of ATF2 was examined by microscopic analysis, and fluorescent images were captured using a charge-coupled device camera mounted on a Nikon TE2000 inverted fluorescence microscope with the DAPI and Texas red filters.

For biochemical subcellular fractionation analysis, cytosolic and nuclear fractions were prepared as described before (33). Cytosolic and nuclear fractions were verified by anti–β-tubulin (Sigma) or anti–histone 3 (Abcam), respectively, in immunoblotting assays. The amounts of ATF2, pCREB, and CREB were determined with anti-ATF2, anti-pCREB, and anti-CREB (Cell Signaling) antibodies. The amounts of ATF2 and pCREB in the cytoplasm or nucleus, respectively, relative to total protein were quantified using ImageJ software.

Transient transfection. To evaluate the effect of ATF2 knockdown, mutant ATF2, or mutant CREB on NED, 60% to 80% confluent LNCaP cells cultured in 10-cm dishes were transfected with the indicated plasmids using FuGENE HD (Roche). Transfected cells were examined for morphologic changes and harvested for determination of expression of NE markers CgA and NSE by immunoblotting 6 d after transfection. The induction of CgA and NSE was quantified using ImageJ software and normalized to β-actin.

IR- and androgen depletion–induced NE-like differentiation in IR-resistant clones. To study IR-induced NED in IR-resistant clones, cells were similarly treated as described above for wild-type LNCaP cells. NE-like cells were visualized by morphologic changes, and the induction of NE markers CgA and NSE and the expression of androgen receptor were determined by immunoblotting with anti-CgA, anti-NSE, and anti–androgen receptor (Santa Cruz Biotechnology) antibodies. To determine the response of IR-resistant clones to androgen depletion treatment, cells were cultured in phenol-free RPMI 1640 supplemented with 10% charcoal-dextran–treated FBS (CD-FBS) for 3 wk and similarly assayed for morphologic changes and the induction of NE markers CgA and NSE. Note that although androgen depletion treatment for 1 wk was sufficient to induce neurite outgrowth, the induction of CgA and NSE expression was barely detectable by immunoblotting even for wild-type LNCaP cells.

Cell viability and growth inhibition assay. Wild-type or IR-resistant clones were cultured in 48-well plates and irradiated with fractionated IR (2 Gy/d) or treated with docetaxel (5 nmol/L) or cultured in phenol-free RPMI 1640 supplemented with 10% CD-FBS for the indicated times. Cell viability for IR- and docetaxel-treated cells was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as described previously (33). Because irradiated cells only showed cell death starting from the 2nd week of irradiation, the cell viability of wild-type LNCaP or IR-resistant clones was determined by comparing to cells that had received 10-Gy irradiation. Because wild-type and IR-resistant clones showed different growth rates and because CD-FBS treatment only inhibited cell growth without inducing cell death, cells cultured in normal FBS were used as controls to first calculate the percentage of growth inhibition (percentage of viable cells in CD-FBS over those in normal FBS), which was subsequently used to calculate the percentage of growth inhibition at different times when compared with cells immediately after treatment (day 0). A Student's t test was applied for statistical analysis.

Results

IR induces NE-like differentiation in LNCaP cells. In an attempt to isolate radiation-resistant clones by following a clinical protocol (70 Gy; ref. 1), we surprisingly found that on 40-Gy irradiation (2 Gy/d, 5 d/wk), the majority of cells (~80%) died whereas cells that survived the treatment displayed the growth of extended neurites (Fig. 1*A*), a NE-like phenotype. Expression of two NE cell markers, CgA and NSE, was significantly induced (Fig. 1*B* and *C*). Similar treatments failed to induce NED in DU145 and PC-3 prostate cancer cells. Consistent with previous reports that NE cells are apoptosis resistant (10, 11), IR-induced NE-like cells were resistant to IR and survived another 3-wk irradiation until the completion of the entire radiation protocol (70 Gy). Addition of the chemotherapeutic agent docetaxel into the IR-induced NE-like cells did not cause any change in cell viability either.

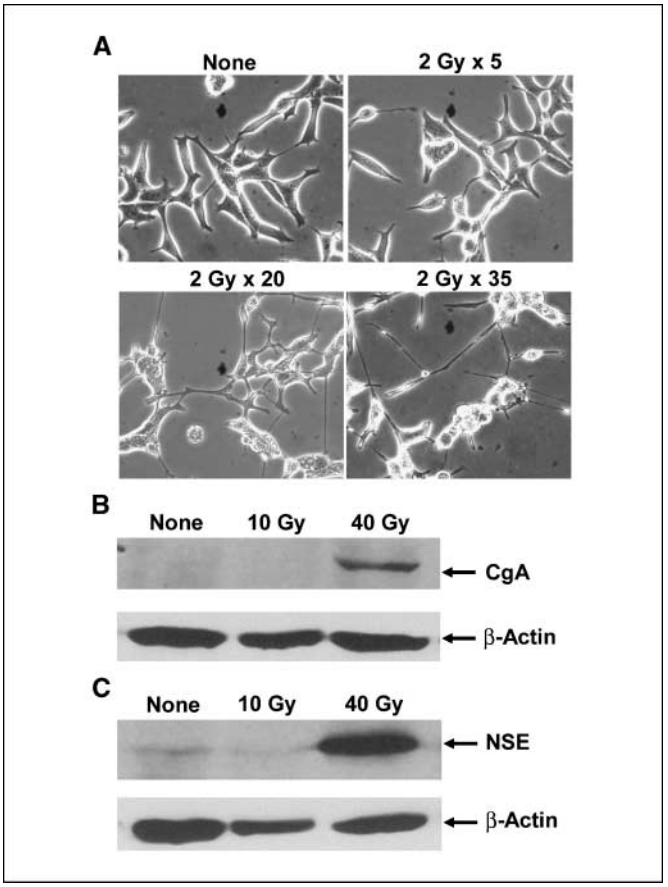

Figure 1. IR induces NE-like differentiation in LNCaP prostate cancer cells. *A,* representative images of cells that received the indicated times of exposures (2 Gy/d, 5 d/wk). Note that cells irradiated 20 times display significant neurite outgrowth and branching. *B* and *C,* immunoblotting of CgA and NSE. Cells that received the indicated dose of radiation were harvested and 20 μg of total protein was used for immunoblotting of CgA and NSE.

IR induces cytoplasmic accumulation of ATF2 and an increase in nuclear pCREB. To determine the subcellular localization of ATF2 in IR-induced NE-like cells, we performed immunostaining and found that ATF2 localization in the cytoplasm was increased compared with nontreated cells (Supplementary Fig. S1*A*). No significant changes in expression and nuclear localization of c-Jun, JunB, and JunD were observed (data not shown), suggesting that the increased cytoplasmic localization of ATF2 is not due to a decrease in Jun proteins to anchor ATF2 in the nucleus (33). In contrast, ATF2 was predominantly localized in the nucleus with some cytoplasmic localization in proliferating LNCaP cells, and treatment with the nuclear export inhibitor leptomycin B (33, 35) increased nuclear localization of ATF2 (Fig. 2*A*). The nuclear sequestration of ATF2 in proliferating LNCaP cells by leptomycin B was also confirmed by subcellular fractionation analysis (data not shown). These results show that ATF2 constantly shuttles between the cytoplasm and nucleus in proliferating LNCaP cells. Consistent with our previous observation that phosphorylation at residues T69 and T71 does not regulate ATF2 subcellular localization (33), the subcellular localization of phospho-ATF2 was similar to that of ATF2 in proliferating and the NE-like cells (data not shown).

To determine whether ATF2 cytoplasmic localization is a consequence or a potential cause of NED, we examined ATF2 subcellular localization at different time points before cells underwent morphologic changes. Irradiation of cells up to five times increased cytoplasmic ATF2 without inducing striking morphologic alterations (Fig. 2*A*). However, treatment of the irradiated cells with leptomycin B failed to induce nuclear accumulation of ATF2 in irradiated cells (Fig. 2*A*), indicating that IR impairs the nuclear import of ATF2. No significant change in ATF2 subcellular localization was observed when irradiated less than five times. Subcellular fractionation analysis showed that IR treatment increased cytoplasmic ATF2 from 24% to 45% of total ATF2 (Fig. 2*B*).

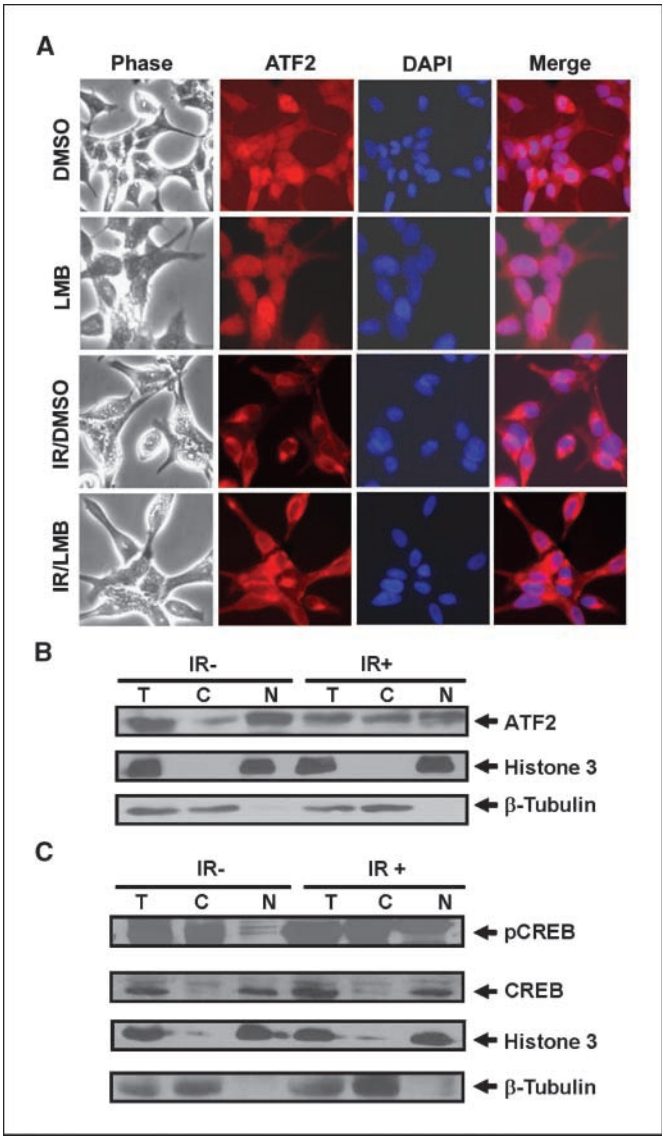

Figure 2. IR induces cytoplasmic accumulation of ATF2 and an increase in nuclear pCREB in LNCaP cells. *A,* LNCaP prostate cancer cells cultured in 12-well plates were treated with DMSO or leptomycin B (*LMB*; 40 ng/mL) overnight or irradiated (2 Gy/d) for 5 d, followed by treatment with DMSO or leptomycin B overnight. Subcellular localization of ATF2 was determined by immunostaining with anti-ATF2 antibody, and DNA in the nucleus was stained with DAPI. *B* and *C,* nonirradiated (*IR−*) or irradiated (2 Gy × 5; *IR+*) LNCaP cells were harvested, and cytosolic, nuclear, and total cellular extracts were prepared. Approximately 20 μg of total cellular extracts (*T*) and an equal portion of cytosolic (*C*) and nuclear (*N*) extracts were used for immunoblotting of ATF2, pCREB, and CREB.

Because CREB regulates transcription of CgA (19), we examined expression and subcellular localization of CREB and pCREB in proliferating and IR-irradiated cells, as we did for ATF2. Because all available pCREB antibodies we tested cross-reacted with phospho-ATF1 and another ~80-kDa cytoplasmic protein (data not shown), we performed subcellular fractionation analysis and determined that IR treatment increased nuclear pCREB from 25% to 49% of the total pCREB (Fig. 2*C*). Unlike pCREB, the nuclear content of CREB was not altered by IR treatment (Fig. 2*C*). Interestingly, pCREB was also detected in the cytoplasm in proliferating LNCaP cells, and IR treatment did not seem to alter the phosphorylation extent of cytoplasmic CREB. IR-induced NE-like cells maintained a high level of pCREB in the nucleus (Supplementary Fig. S1*B*). Taken together, these results show that IR-induced cytoplasmic accumulation of ATF2 and increase in nuclear pCREB occur before cells undergo differentiation.

CREB and ATF2 play opposing roles in NE-like differentiation. The IR-induced cytoplasmic accumulation of ATF2 and increase in nuclear pCREB prompted us to test the hypothesis that nuclear CREB and ATF2 may play opposing roles in NED. Indeed, 50% knockdown of ATF2 resulted in a NE-like morphologic change (Supplementary Fig. S2*A*) and a 1.6-fold induction of NSE (Fig. 3*A*). No induction of CgA was observed (data not shown). In contrast, transient expression of VP16-bCREB, a constitutively activated and nuclear-localized mutant of CREB (36, 37), induced a NE-like morphologic change (Supplementary Fig. S2*B*) and increased CgA and NSE expression by 2- to 3-fold (Fig. 3*B*). However, overexpression of a constitutively nuclear-localized ATF2 (nATF2), which has a nuclear localization signal from the large T antigen of SV40 fused to the NH_2 terminus of ATF2 as others did (34), inhibited VP16-bCREB–mediated morphologic changes and the induction of NSE. To determine whether increased cytoplasmic accumulation of endogenous ATF2 can induce NED, we overexpressed a constitutively cytoplasmic-localized ATF2 (cATF2), which lacks the two nuclear localization signals (33), in LNCaP cells. Because ATF2 homodimerization impairs ATF2 nuclear import (33), overexpression of cATF2 increased cytoplasmic localization of ATF2 to ~50% of total ATF2 (data not shown). Indeed, cATF2, but not nATF2, induced neurite outgrowth (Supplementary Fig. S2*C*) and a 5.4-fold increase in NSE expression (Fig. 3*C*). No induction of CgA by cATF2 or nATF2 was observed. Transiently expressed cATF2-Venus and nATF2-Venus were predominantly localized to the cytoplasm and nucleus, respectively (Supplementary Fig. S2*D*). Immunoblotting analysis confirmed the exogenous expression of VP16-bCREB, cATF2-Venus, and nATF2-Venus in these experiments (data not shown). Knockdown of ATF2 or expression of cATF2 had no effect on the localization and amount of pCREB, and overexpression of VP16-bCREB did not alter subcellular localization of ATF2 (data not shown). Taken together, these results support the hypothesis that CREB and ATF2 play opposing roles in NED.

Stable expression of a nonphosphorylatable CREB or nATF2 inhibits IR-induced NE-like differentiation. To further determine the role of CREB and ATF2 in IR-induced NED, we established tetracycline-inducible stable cell lines that express nATF2 or a nonphosphorylatable CREB (CREB-S133A), which has been used as a dominant negative mutant form of CREB (13, 15). In the absence of tetracycline, these stable cell lines exhibited normal morphology like vector-only cells (Fig. 4*A*). However, addition of tetracycline significantly induced expression of CREB-S133A or nATF2 (Fig. 4*B*) and reduced the percentage of cells displaying extended neurites in response to irradiation (Fig. 4*C*).

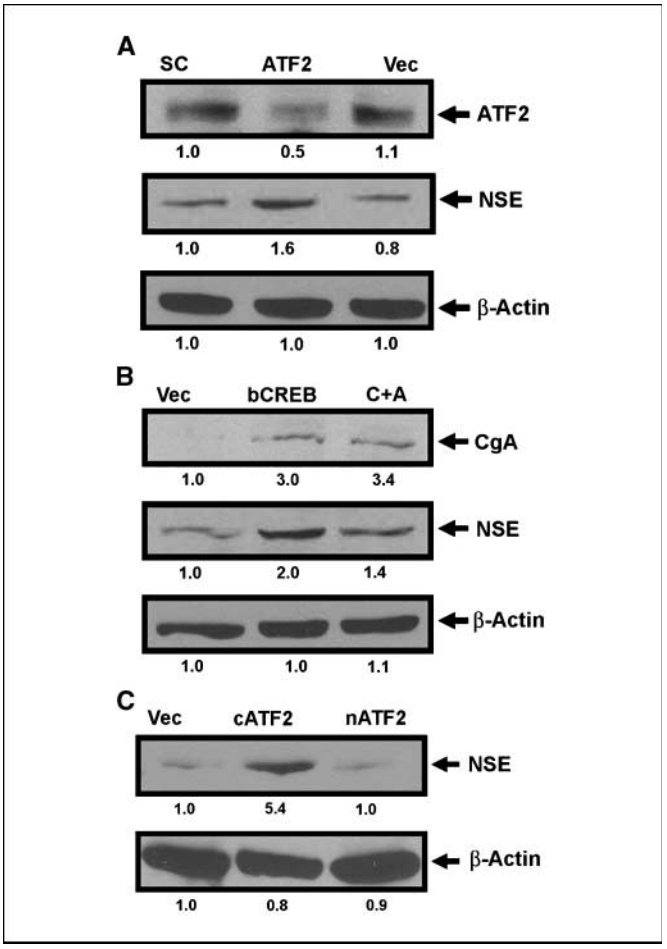

Figure 3. ATF2 and CREB play opposing roles in NE-like differentiation. *A,* immunoblotting analysis of ATF2 and NSE expression from LNCaP cells transfected with siRNA constructs for scrambled sequences (*SC*), ATF2 siRNA (*ATF2*), or pSUPER vector only (*Vec*). *B,* immunoblotting analysis of CgA and NSE from LNCaP cells transfected with the vector control (*Vec*), the plasmid encoding VP16-bCREB (*bCREB*), or cotransfected with plasmids encoding VP16-bCREB and nATF2 (*C+A*). *C,* immunoblotting analysis of NSE from LNCaP cells transfected with the vector control (*Vec*) or the plasmid encoding cATF2 or nATF2. The number below each lane is the quantified fold change when compared with the first lane.

Interestingly, induction of CgA and NSE by IR was inhibited by nATF2, but not by CREB-S133A (Fig. 4*D*). These results further support the conclusion that nuclear ATF2 and pCREB play different roles in IR-induced neurite outgrowth.

To determine the relationship between the expression of nATF2 and IR-induced phosphorylation of CREB and the relationship between the expression of CREB-S133A and the subcellular localization of ATF2, we irradiated cells for 5 days while constantly inducing expression of nATF2 or CREB-S133A. Expression of CREB-S133A did not affect IR-induced cytoplasmic localization of ATF2 (data not shown), whereas expression of nATF2 significantly inhibited IR-induced phosphorylation of CREB (Supplementary Fig. S3). However, expression of nATF2 only did not affect phosphorylation of CREB in the absence of IR (data not shown). These results suggest that IR-induced cytoplasmic sequestration of ATF2 may be a prerequisite for IR-induced phosphorylation of CREB and the subsequent NE-like differentiation.

IR-induced NE-like differentiation is reversible, and dedifferentiated cells lose NE-like properties. Because cAMP-induced

NE-like cells are reversible (12), we sought to determine whether IR-induced NE-like cells are also reversible. We irradiated cells for 4 weeks (40 Gy) to allow all surviving cells to differentiate into NE-like cells and then waited for the growth of any cells that were reversible. Although differentiated NE-like cells were maintained without obvious cell death or growth for the first 2 months, we isolated three independent clones 3 months after the completion of the irradiation. We named these clones LNCaP-IRR1 (IRR refers to IR resistant), LNCaP-IRR2, and LNCaP-IRR3. These IR-resistant cells showed similar morphology to wild-type LNCaP cells (Supplementary Fig. S4). All three clones lost CgA and NSE expression but retained levels of androgen receptor comparable to wild-type LNCaP cells (Fig. 5*A*), suggesting that these clones have lost their NE-like cell properties.

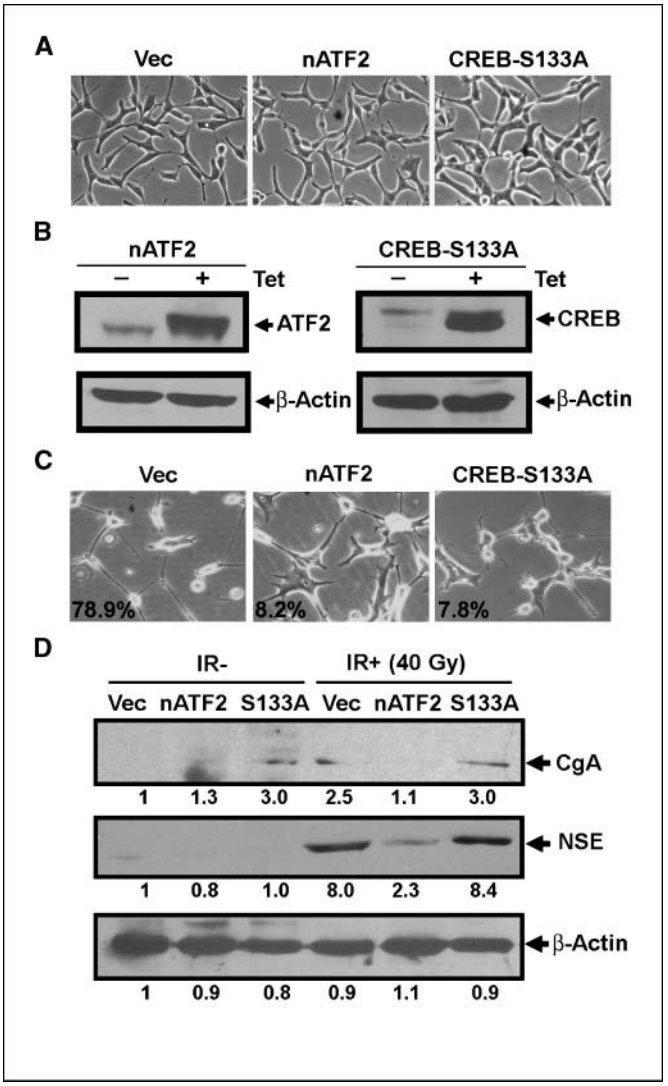

Figure 4. Inhibition of IR-induced NE-like differentiation by dominant negative CREB and nATF2. *A,* representative images of stable cell lines that have pcDNA4/TO (*Vec*), pcDNA4-TO-Flag-nATF2 (*nATF2*), or pcDNA4/TO-HA-CREB-S133A (*CREB-S133A*) integrated. *B,* immunoblotting analysis of induced nATF2 and CREB-S133A by tetracycline. Total cell lysates were prepared 3 d after the induction, and Flag-nATF2 and HA-CREB-S133A were detected with anti-ATF2 and anti-HA antibodies, respectively. *C,* representative images acquired from stable cell lines that received 40-Gy irradiation in the presence of tetracycline. The number indicates the percentage of cells showing extended neurites. *D,* immunoblotting analysis of CgA and NSE from experiments in *C*. The number below each lane is the quantified fold change when compared with the first lane.

To determine whether these IR-resistant clones can still be induced to redifferentiation, we irradiated them at 40 Gy and examined for morphologic changes and the induction of CgA and NSE. Whereas all three clones exhibited extended neurite outgrowth (Supplementary Fig. S5*A*), the induction of CgA and NSE was completely abrogated (Fig. 5*A*). Interestingly, androgen receptor expression in LNCaP-IRR2 clone was significantly inhibited like in parental cells whereas androgen receptor expression in LNCaP-IRR1 and LNCaP-IRR3 cells was only slightly attenuated. These distinct responses to IR treatment suggest that these three IR-resistant clones are likely heterogeneous. To determine how these clones respond to androgen depletion treatment, we treated cells in phenol-free medium supplemented with 10% CD-FBS for 3 weeks. Whereas LNCaP-IRR1 and LNCaP-IRR3 cells exhibited extended neurite outgrowth, LNCaP-IRR2 cells showed only short neurites (Supplementary Fig. S5*B*). Interestingly, an induction of CgA expression by CD-FBS similar to parental cells was observed in LNCaP-IRR2; no induction was observed in LNCaP-IRR1; and a significantly attenuated induction was seen in LNCaP-IRR3 cells (Fig. 5*B*). On the contrary, the induction of NSE in LNCaP-IRR2 was abolished, whereas LNCaP-IRR1 and IRR3 responded to the treatment to some extent. Like the parental cells, however, the expression of androgen receptor in all three clones was significantly down-regulated by the CD-FBS treatment. Taken together, these results suggest that the three IR-resistant clones are heterogeneous and likely have distinct molecular defects in their responses to IR and androgen depletion treatments.

IR-resistant and dedifferentiated cells acquire cross-resistance to therapy. To explore the potential implication of dedifferentiated cells in prostate cancer progression, we examined their response to radiation, the chemotherapeutic agent docetaxel (38), and androgen depletion treatments. Like the parental LNCaP cells, all three clones stopped growth during the 1st week of irradiation (10 Gy) and no cell death was observed (Fig. 6*A*). During the 2nd week of irradiation, however, all three clones showed significantly reduced cell death when compared with the parental cells. Interestingly, all three IR-resistant cells began to resume growth during the 3rd week of irradiation whereas the parental cells did not show obvious growth or death as all surviving cells differentiated into NE-like cells. Similar to their response to IR treatment, all three IR-resistant clones were resistant to cell death induced by the chemotherapeutic agent docetaxel (Fig. 6*B*), as well as to growth inhibition on androgen depletion (Fig. 6*C*). These results suggest that IR-induced NE-like cells have the potential to dedifferentiate back into a proliferating state with the acquisition of cross-resistance to radiotherapy, chemotherapy, and hormonal therapy.

Discussion

NE-like cells are implicated in prostate cancer progression, androgen-independent growth, and poor prognosis (3–6, 39, 40). Because androgen ablation treatment can induce NED *in vitro* and *in vivo* (3–6), it has been proposed that the presence of NE-like cells may contribute to androgen-independent growth, a critical factor leading to the failure of current prostate cancer therapy. We present here the first evidence that in addition to androgen ablation, IR also induces NED in the prostate cancer cell line LNCaP.

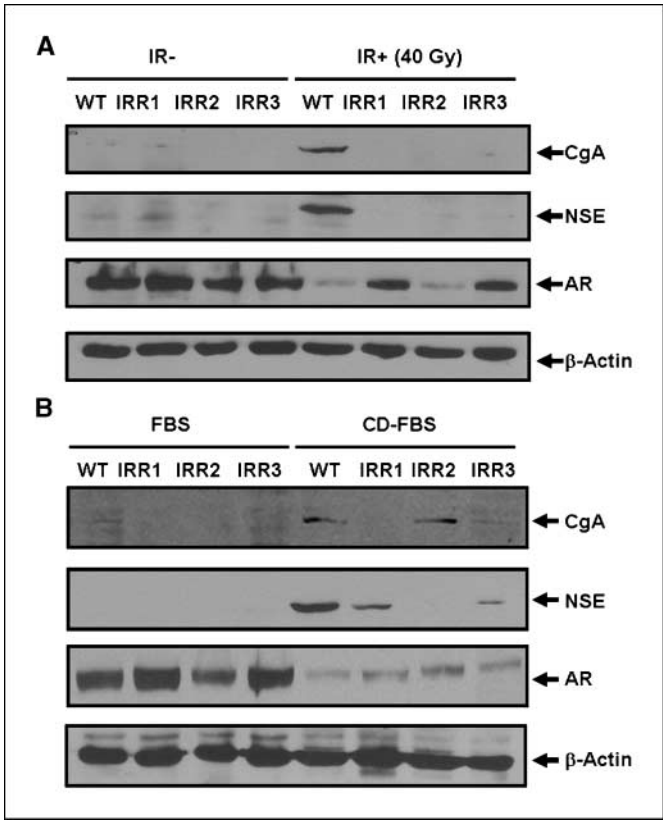

Figure 5. Response of IR-resistant clones to IR- and androgen depletion–induced NE-like redifferentiation. *A,* wild-type LNCaP (*WT*) and the indicated IR-resistant clones were subjected to fractionated IR (40 Gy), and the induction of CgA and NSE as well as the expression of androgen receptor (*AR*) was compared with that of nonirradiated cells. *B,* wild-type LNCaP and IR-resistant clones were cultured in medium supplemented with 10% FBS or CD-FBS for 3 wk, and the induction of CgA and NSE as well as the expression of androgen receptor was determined by immunoblotting.

Significantly, IR-induced NED is reversible, and dedifferentiated cells have lost the NE-like properties. However, all isolated three IR-resistant clones derived from dedifferentiated cells are cross-resistant to radiation, docetaxel, and androgen depletion treatments. These findings, along with other reports (41–46), strongly suggest that radiation- or hormonal therapy–induced NED may represent a common pathway by which cancer cells survive treatment and contribute to prostate cancer recurrence.

Although it has been reported that signal transducer and activator of transcription-3 (47) and β-catenin (48) can mediate IL-6– and androgen depletion–induced NED in prostate cancer cells, respectively, it remains largely unexplored how the switch from proliferation to differentiation is turned on at the transcriptional level. Several pieces of evidence presented in this work show that CREB functions as a transcriptional activator and ATF2 acts as a transcriptional repressor of NED. First, IR induced cytoplasmic accumulation of ATF2 and increased nuclear pCREB. Second, knockdown of ATF2 or overexpression of VP16-bCREB induced NED. Third, overexpression of nATF2 inhibited NED induced by VP16-bCREB, whereas overexpression of cATF2 induced NED. Last, stable expression of CREB-S133A or nATF2 inhibited IR-induced NED.

The transcriptional regulation of cAMP responsive element–containing genes by ATF2 and CREB is dependent on individual genes. For example, the insulin promoter contains one cAMP responsive element–binding site, and both ATF2 and CREB can bind it. However, ATF2 activates the transcription of insulin, whereas CREB inhibits it (31). In the present work, we also observed that overexpression of VP16-bCREB increased expression of endogenous CgA and NSE, whereas overexpression of nATF2 inhibited VP16-bCREB–induced expression of NSE, but not CgA. Likewise, knockdown of ATF2 or overexpression of cATF2 increased expression of NSE, but not CgA. These results support the notion that the effect of CREB and ATF2 on target gene transcription is dependent on gene context. Although VP16-bCREB can induce CgA and NSE expression (Fig. 3*B*), stable expression of nATF2, but not CREB-S133A, inhibited IR-induced expression of CgA and NSE (Fig. 4*D*). Despite the fact that the CREB-S133A–expressing stable cell line seems to have a basal level of CgA

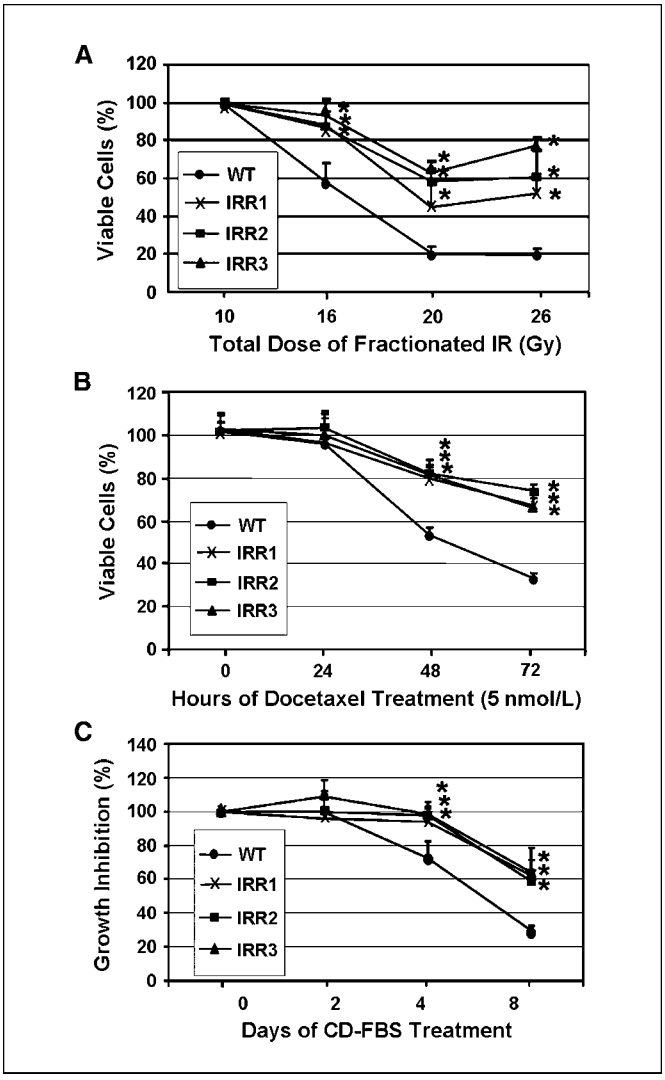

Figure 6. Cross-resistance of IR-resistant clones to therapeutic treatments. *A,* wild-type LNCaP and the indicated IR-resistant clones were cultured in 48-well plates and subjected to fractionated IR for the indicated doses. Cell viability was determined 1 d after the indicated irradiation as the percentage of viable cells that received 10-Gy irradiation. *B,* cells were treated with docetaxel for the indicated time and cell viability was determined as the percentage of viable cells at 0 h. *C,* cells were cultured in 10% FBS or CD-FBS for the indicated time and the inhibition of cell growth by CD-FBS was determined as described in Materials and Methods. *, $P < 0.01$, compared with wild-type LNCaP cells.

expression in the absence of tetracycline, which is likely due to leaky expression of CREB-S133A, induction of CREB-S133A by tetracycline did not alter the CgA expression in response to IR (Fig. 4*D*). Given that overexpression of VP16-bCREB induced expression of both CgA and NSE (Fig. 3*B*), these observations suggest that CREB is not responsible for IR-induced CgA and NSE expression. Alternatively, phosphorylation of CREB at different sites (21) may contribute to IR-induced CgA and NSE expression. Future studies are needed to distinguish these two possibilities. Interestingly, overexpression of CREB-S133A and nATF2 did not inhibit the growth of shorter neurites but rather inhibited the elongation of neurites (Fig. 4*C*). Consistent with a role of CREB in neurite elongation in hippocampal neurons (49), it is likely that CREB and ATF2 may oppose each other in irradiated LNCaP cells to regulate transcription of target genes essential for neurite elongation, one of the phases during neuritogenesis (50). Further identification of the target genes will provide insight into the molecular mechanisms by which CREB and ATF2 play opposing roles in IR-induced NED. Because expression of nATF2 inhibited IR-induced phosphoryaltion of CREB (Supplementary Fig. S3), it is possible that nuclear ATF2 may also antagonize an upstream signaling pathway that contributes to IR-induced phosphorylation of CREB. It will be interesting to determine whether this effect is independent of or dependent on ATF2 transcriptional activity. In addition, identification of cell signaling that regulates cytoplasmic accumulation of ATF2 and phosphorylation of CREB will provide opportunities to develop novel therapeutics for prostate cancer.

The finding that IR can induce NED is clinically important, given that ~10% to 60% of patients treated with radiation therapy experience recurrent tumors (2). Although a detailed and well-controlled examination of NE-like cells in recurrent tumors would shed light on our *in vitro* findings here, the fact that patients who have biochemical recurrence after radiotherapy normally do not undergo surgery or even biopsy prevents us from performing this type of study. In addition, the transient nature of NE-like cells may also not allow us to find a causative link between radiation therapy and the induction of NED in patients. We are therefore currently performing longitudinal analyses to evaluate the effect of radiation therapy on NED and its contribution to tumor recurrence in xenograft nude mouse prostate cancer models.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Legends to Supplementary Figures

Supplementary SFig. 1. **IR induces cytoplasmic accumulation of ATF2 and nuclear accumulation of pCREB in NE-like cells.** (*A*). LNCaP cells cultured in 10 cm dishes were irradiated with fractionated IR (2 Gy/day, 5 days/week) for four weeks to induce NE-like differentiation. Subcellular localization of ATF2 in NE-like cells was determined using immunostaining. DAPI staining was used to mark the nucleus. Subcellular localization of ATF2 in proliferating LNCaP cells was shown in Fig. 2A. (*B*). LNCaP cells were similarly treated as described in (*A*) and cytosolic and nuclear extracts were prepared. For the purpose of comparison, non-irradiated cells (-) and cells that received 5 times of exposure to IR (10 Gy) were also included. Note that only 10 μg of cytosolic extract and the equal portion of nuclear extract were loaded for immunoblotting of pCREB.

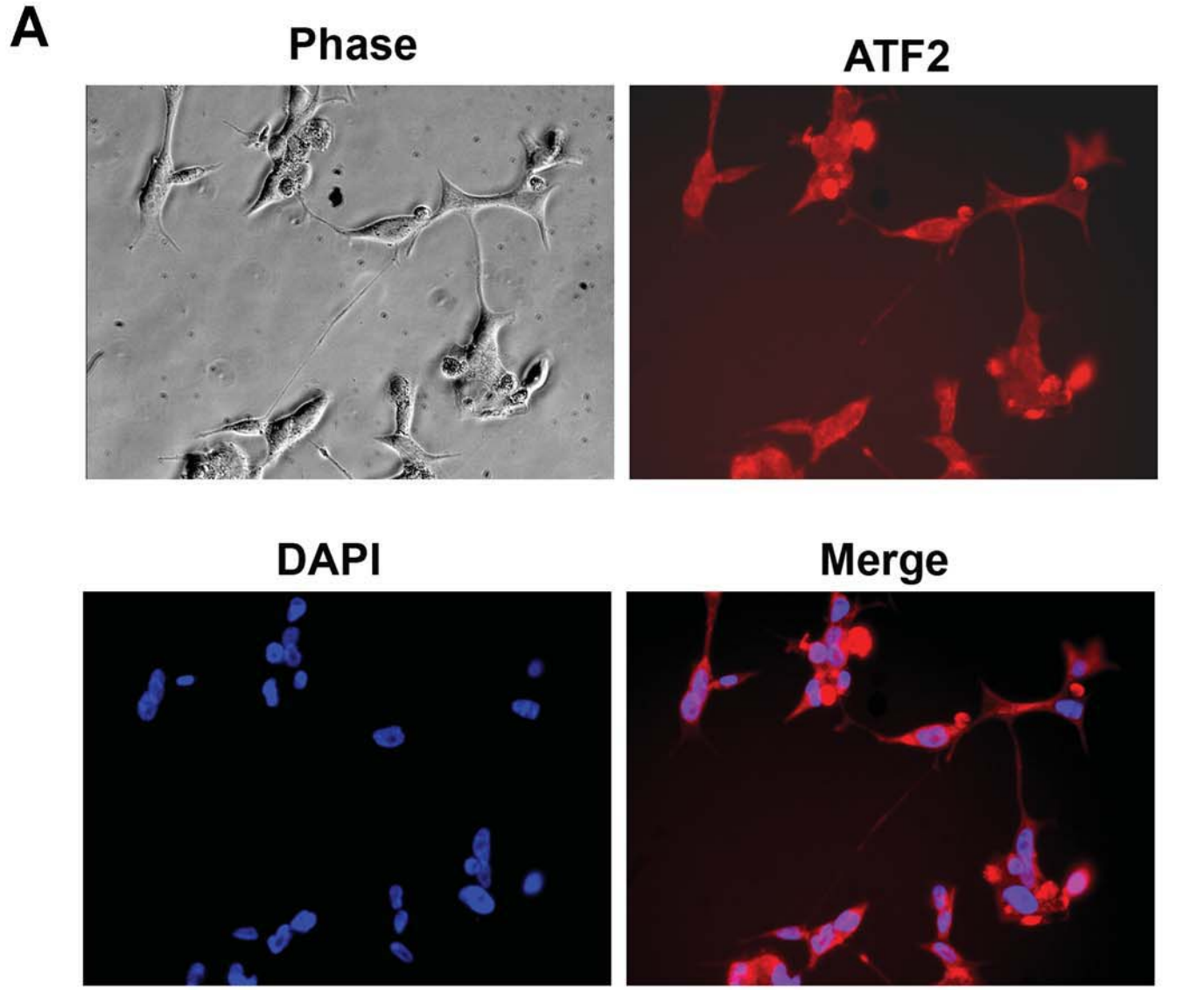
Supplementary SFig. 2 ATF2 and CREB play an opposing role in NE-like differentiation. Shown are representative images acquired from cells transfected with plasmids encoding the indicated siRNA constructs (*A*), mutant CREB (*B*), or mutant ATF2 (*C*) as presented in Fig. 3. (*D*) Shown are subcellular localization of cATF2 and nATF2 as Venus fusions.

Supplementary SFig. 3. Effect of nATF2 on IR-induced phosphorylation of CREB. The LNCaP cells that stably express inducible nATF2 were cultured in 10 cm dishes and subjected to IR (2 Gy/day) for five days while nATF2 expression was constantly induced by tetracycline (Tet +) or not induced (Tet -). Irradiated cells were harvested, and

cytosolic (C) and nuclear (N) fractions were prepared. The amount of pCREB, CREB, and ATF2 in the cytosolic and nuclear fractions was determined using immunoblotting.

Supplementary SFig. 4. Morphology of wild-type LNCaP (WT) and the indicated isolated IR-resistant clones from dedifferentiated NE-like cells.

Supplementary SFig. 5. IR- and androgen depletion-induced NE-like morphological changes in wild-type LNCaP (WT) cells and IR-resistant clones. (*A*) Shown are representative images of irradiated cells acquired at the end of 40 Gy-irradiation. (*B*) Shown are representative images of cells acquired at the end of three-week treatment with C/D-FBS.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pCREB
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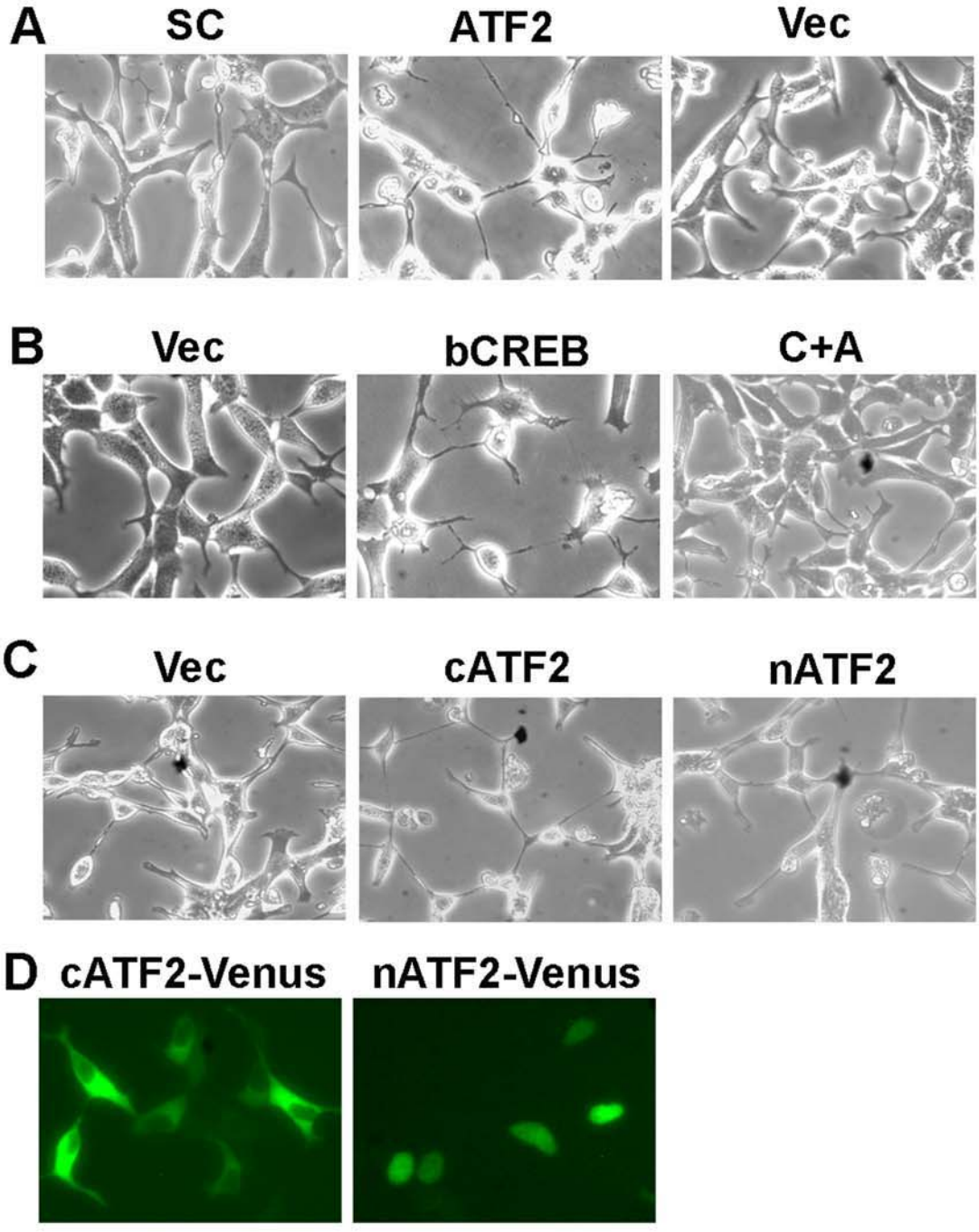
β–tubulin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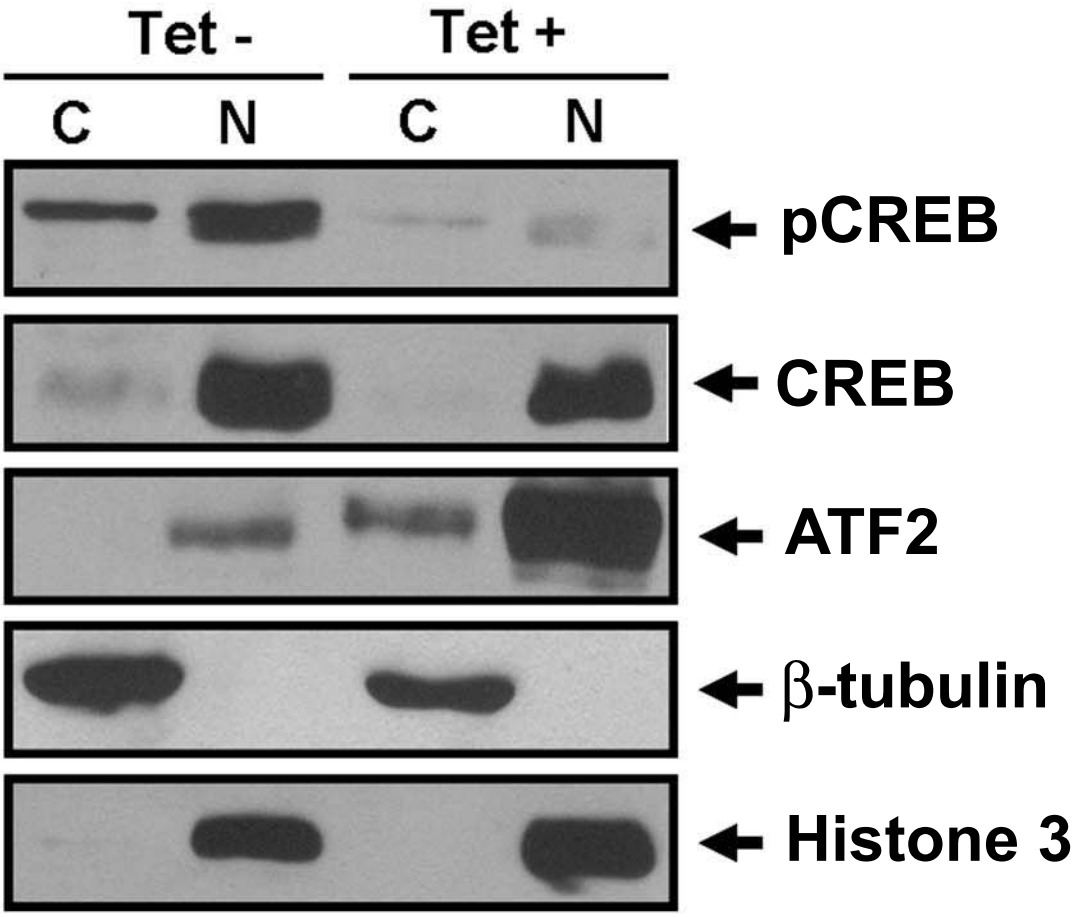
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CREB
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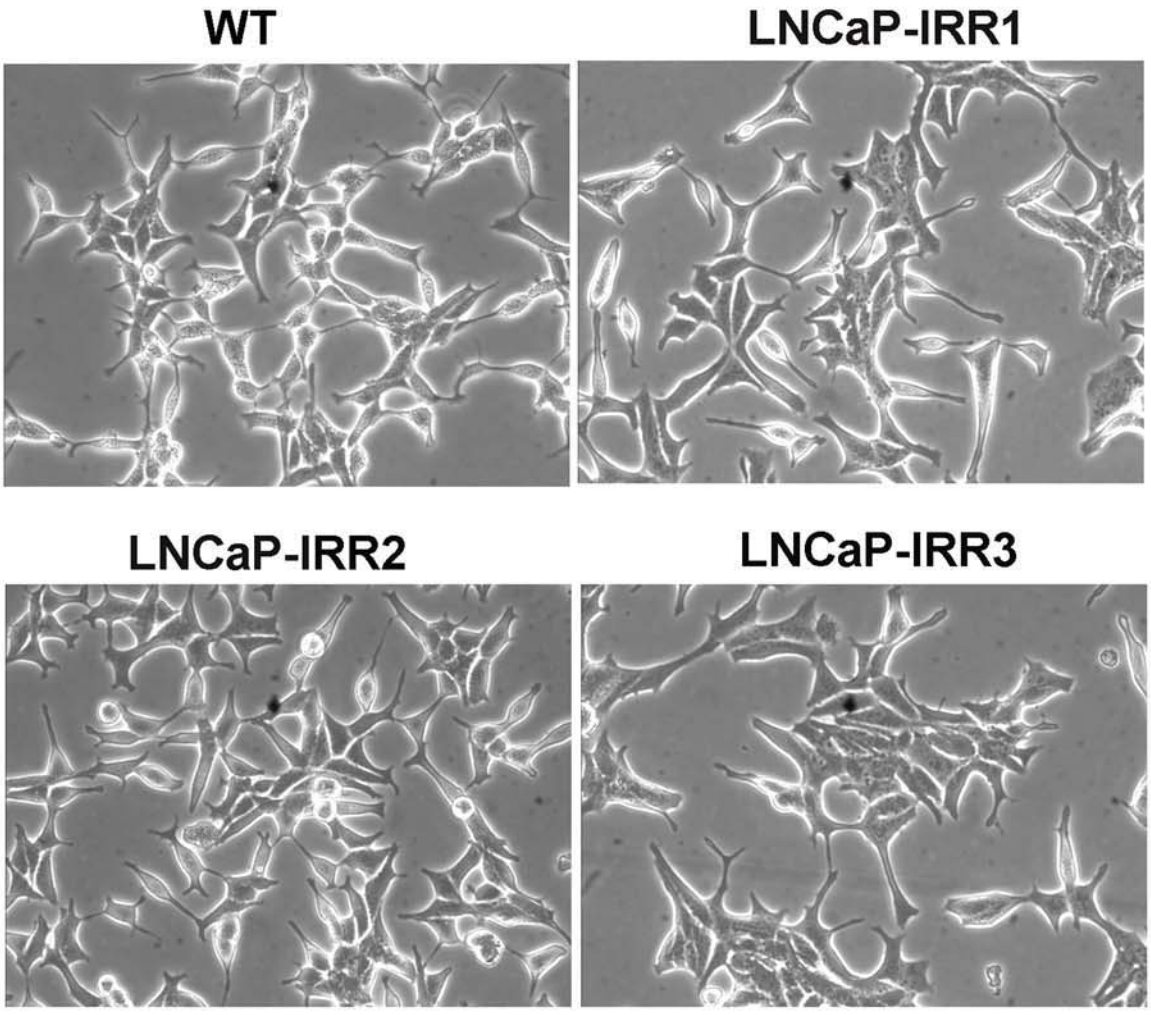
Histone 3


WT
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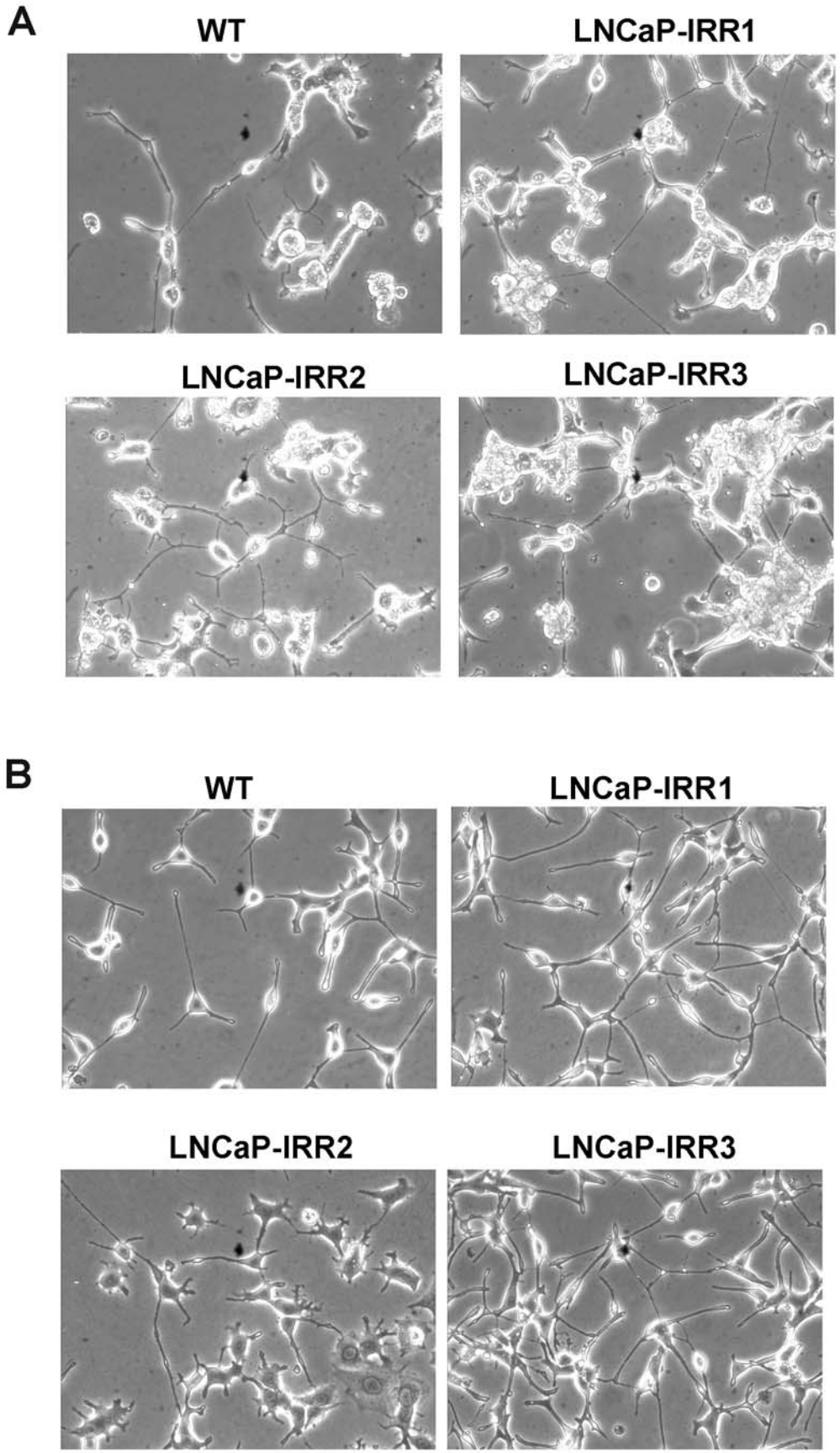
LNCaP-IRR2
LNCaP-IRR3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B
WT
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