Type 1 Insulin-like Growth Factor Receptor Translocates to the Nucleus of Human Tumor Cells

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Abstract

The type 1 insulin-like growth factor receptor (IGF-1R) is a transmembrane glycoprotein composed of two extracellular α subunits and two β subunits with tyrosine kinase activity. The IGF-1R is frequently upregulated in cancers and signals from the cell surface to promote proliferation and cell survival. Recent attention has focused on the IGF-1R as a target for cancer treatment. Here, we report that the nuclei of human tumor cells contain IGF-1R, detectable using multiple antibodies to α- and β-subunit domains. Cell-surface IGF-1R translocates to the nucleus following clathrin-mediated endocytosis, regulated by IGF levels. The IGF-1R is unusual among transmembrane receptors that undergo nuclear import, in that both α and β subunits traffic to the nucleus. Nuclear IGF-1R is phosphorylated in response to ligand and undergoes IGF-induced interaction with chromatin, suggesting direct engagement in transcriptional regulation. The IGF dependence of these phenomena indicates a requirement for the receptor kinase, and indeed, IGF-1R nuclear import and chromatin binding can be blocked by a novel IGF-1R kinase inhibitor. Nuclear IGF-1R is detectable in primary renal cancer cells, formalin-fixed tumors, preinvasive lesions in the breast, and nonmalignant tissues characterized by a high proliferation rate. In clear cell renal cancer, nuclear IGF-1R is associated with adverse prognosis. Our findings suggest that IGF-1R nuclear import has biological significance, may contribute directly to IGF-1R function, and may influence the efficacy of IGF-1R inhibitory drugs. *Cancer Res; 70(16); 6412–9. ©2010 AACR.*

Introduction

The type 1 insulin-like growth factor receptor (IGF-1R) mediates proliferation and cell survival and is recognized as an attractive cancer treatment target (1). Following cotranslational insertion into the endoplasmic reticulum (ER) as a 220-kDa proreceptor, the IGF-1R is cleaved in the trans-Golgi network to generate mature α subunits (135 kDa) and β subunits (98 kDa) linked by disulfide bonds (2). After trafficking to the plasma membrane, IGF-1Rs are activated by IGFs and then internalized and degraded or recycled to the cell surface (3, 4). Whereas other receptor tyrosine kinases (RTK) are known to undergo nuclear translocation (5–8), nuclear IGF-1R has not been reported in human cancers, although it was detected in hamster kidney cells (9). Building on our studies of IGF signaling in prostate cancer and renal cell cancer (RCC; refs. 10–13), we hypothesized that the IGF-1R undergoes nuclear translocation in these tumors.

Materials and Methods

Human DU145 prostate cancer, 786-0/EV RCC, and MCF7 breast cancer cells were from Cancer Research UK. IGF-1R–null murine fibroblasts (R– cells) and isogenic R+ cells expressing human IGF-1R were from Renato Baserga (Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA). Primary RCC cultures were generated by disaggregation of fresh tumors and stained for pancytokeratin (Abcam). Cells were transfected with IGF-1R (#SI00017521), caveolin (#SI00027720), or control (#1022076) siRNAs (Qiagen) using Oligofectamine (Invitrogen). The IGF-1R antibody MAB391 was from R&D Systems. AZ12253801 (from Elizabeth Anderson, AstraZeneca, United Kingdom) is an ATP-competitive IGF-1R tyrosine kinase inhibitor that shows ∼10-fold selectivity over the insulin receptor. The IC_{50} values for inhibition of IGF-1R and insulin receptor phosphorylation *in vitro* are 2.1 and 19 nmol/L, respectively. The IC_{50} for inhibition of IGF-1R–driven proliferation in 3T3 mouse fibroblasts transfected with human IGF-1R is 17 nmol/L, whereas the IC_{50} for epidermal growth factor receptor (EGFR)–driven proliferation is 440 nmol/L. AZ12253801 has been tested against a wide range of other relevant kinases, where IC_{50}s are generally >1 μmol/L or the compound has little or no inhibitory activity at 10 μmol/L.

Immunofluorescence

Cells were cultured in complete medium or serum starved overnight and treated with long-R3 IGF-I (SAFC Biosciences), IGF-II, insulin (Sigma-Aldrich), or solvent. Some cultures were pretreated with solvent (DMSO), 300 nmol/L dibenzazepine (Calbiochem), 300 μmol/L dansylcadaverine (Sigma-Aldrich), 30 μmol/L dynasore (Sigma-Aldrich), or AZ12253801. Immunostaining used antibodies to IGF-1Rβ COOH terminus [3027, Cell Signaling Technology (CST)], IGF-1Rβ NH₂ terminus (H-60, Santa Cruz), IGF-1Rα [24-31 (Ken Siddle, Department of Clinical Biochemistry, University of Cambridge, Cambridge, UK) or αIR3 (#GR11L, Calbiochem)], calnexin, nucleolin, or RNA polymerase II (Abcam). Images were acquired on a LSM 510 confocal microscope (Zeiss). Photomicrographs show mid-slice confocal images through the nucleus, ×63 magnification unless stated otherwise. Fluorescence was quantified using ImageJ software in 20 to 30 cells for each condition, and statistical analysis used GraphPad Prism v5.

Cell fractionation, immunoblotting, and immunoprecipitation

Whole-cell extracts were prepared in radioimmunoprecipitation assay buffer (14). Nuclear extraction used nuclear extraction reagents (Panomics) to disrupt cells in hypotonic buffer A, and nuclear proteins were released with buffer B (high salt with detergent). Whole-cell, non-nuclear, and nuclear fractions and chromatin extracts were analyzed by SDS-PAGE and immunoblotting for IGF-1Rα (Santa Cruz), IGF-1Rβ (3027, CST), phosphorylated IGF-1R (Y1135-6, CST), lamin, calnexin (Abcam), golgin-84 (BD Biosciences), EpCAM (clone AUA1, Cancer Research UK), β-tubulin (Sigma-Aldrich), and Hes1 (gift of Dr. Tatsuo Sudo, Toray Industries, Kamakura, Japan). Extracts were immunoprecipitated with IGF-1Rβ antibody (3027, CST) or rabbit IgGs (Sigma-Aldrich); see Supplementary Data.

Immunohistochemistry

Human tissue was used under National Research Ethics studies 04/Q1606/96, 07/H0606/120, and 09/H0606/5. Formalin-fixed whole mount and tissue microarray (TMA) sections were immunostained for IGF-1Rβ (3027, CST) and IGF-1Rα (24-31). IGF-1R intensity and distribution were scored as described (10, 13, 15). Contingency tables were analyzed using Pearson's χ^2 test to assess relationships between IGF-1R and clinical parameters. Survival was measured from nephrectomy to death or last follow-up, and survival curves were estimated by the Kaplan-Meier method. Prognostic factors were evaluated in multivariate analyses by Cox proportional hazards regression. These analyses used the STATA package v11.0 (Stata Corporation).

Results and Discussion

We hypothesized that the IGF-1R undergoes nuclear import and indeed could detect intracellular IGF-1R in prostate cancer (Fig. 1A), RCC, and breast cancer cells (Supplementary Fig. S1). Intracellular IGF-1R was attenuated by *IGF-1R* gene silencing, was not wholly attributable to receptor within

ER, and seemed to overlie the nucleus, sparing the nucleoli (Fig. 1A and B). Detection of nuclear receptor was unrelated to IGF-1R levels per se: IGF-1R–overexpressing R+ cells contained negligible nuclear IGF-1R (Supplementary Fig. S1). Nuclear translocation of other RTKs can involve import of full-length receptor or enzymatic release of receptor intracellular domains, each process initiated when receptor is engaged by ligand (5, 7, 8). We found that serum-starved cells showed prominent membrane IGF-1R that diminished with IGF-I treatment (Fig. 1C), consistent with receptor internalization and degradation (3). Persisting IGF-1R showed evidence of nuclear accumulation 15 to 60 minutes after addition of 30 to 50 nmol/L IGF-I (Fig. 1C and D; Supplementary Fig. S2). IGF-1R nuclear import was also enhanced by IGF-II, but only modestly by insulin, correlating with the magnitude of ligand-induced receptor phosphorylation (Supplementary Fig. S3) and with the known affinity of these ligands for IGF-1R (2).

The IGF-1R β-subunit is reportedly a substrate for γ-secretase, liberating 50- to 52-kDa intracellular domains in R+ cells (16). However, IGF-1R distribution was unaffected by γ-secretase inhibition in prostate cancer cells (Fig. 2A), and we detected full-length IGF-1Rα and IGF-1Rβ in nuclear extract (Fig. 2B). Furthermore, nuclear IGF-1R was detectable using antibodies to β-subunit extracellular domain (Supplementary Fig. S4A) and α-subunit, which also showed IGF-induced nuclear accumulation (Fig. 2C; Supplementary Fig. S4B). Therefore, our data do not support γ-secretase–dependent cleavage, but instead suggest a model in which full-length IGF-1R translocates to the nucleus. Other full-length receptors known to undergo nuclear translocation are monomers (5, 8); to our knowledge, IGF-1R is the only example of a receptor that traffics as multiple subunits to the nucleus.

The IGF-1R undergoes both caveolin- and clathrin-mediated endocytosis (4, 17). Consistent with the contribution of the latter to EGFR nuclear import (5, 18), nuclear IGF-1R translocation was inhibited by dansylcadaverine ($P < 0.001$) and the dynamin-1 inhibitor dynasore ($P < 0.05$), inhibitors of clathrin-dependent endocytosis, and by bafilomycin A1 ($P < 0.001$), which blocks endosomal acidification, but not by caveolin-1 depletion (Fig. 2D; Supplementary Fig. S5). Post-endosomal EGFR trafficking involves translocation to the ER, removal from the lipid bilayer by association with a component of the Sec61 translocon (6), and nuclear import in complex with importins (5, 18). The IGF-1R lacks a canonical nuclear localization sequence, and we could not detect binding to importin-β (not shown). Neither was there evidence of interaction between nuclear IGF-1R and the adaptor protein insulin-receptor substrate 1, which can undergo nuclear import (19), but is predominantly cytoplasmic in DU145 cells (not shown). While our article was under review, Sehat and colleagues reported that the IGF-1R undergoes nuclear translocation and showed that this is regulated by SUMOylation, not previously known to influence RTK localization (20).

We noted that IGFs and insulin induced IGF-1R nuclear import in proportion to their ability to activate the receptor, and nuclear IGF-1Rβ was phosphorylated in response to ligand
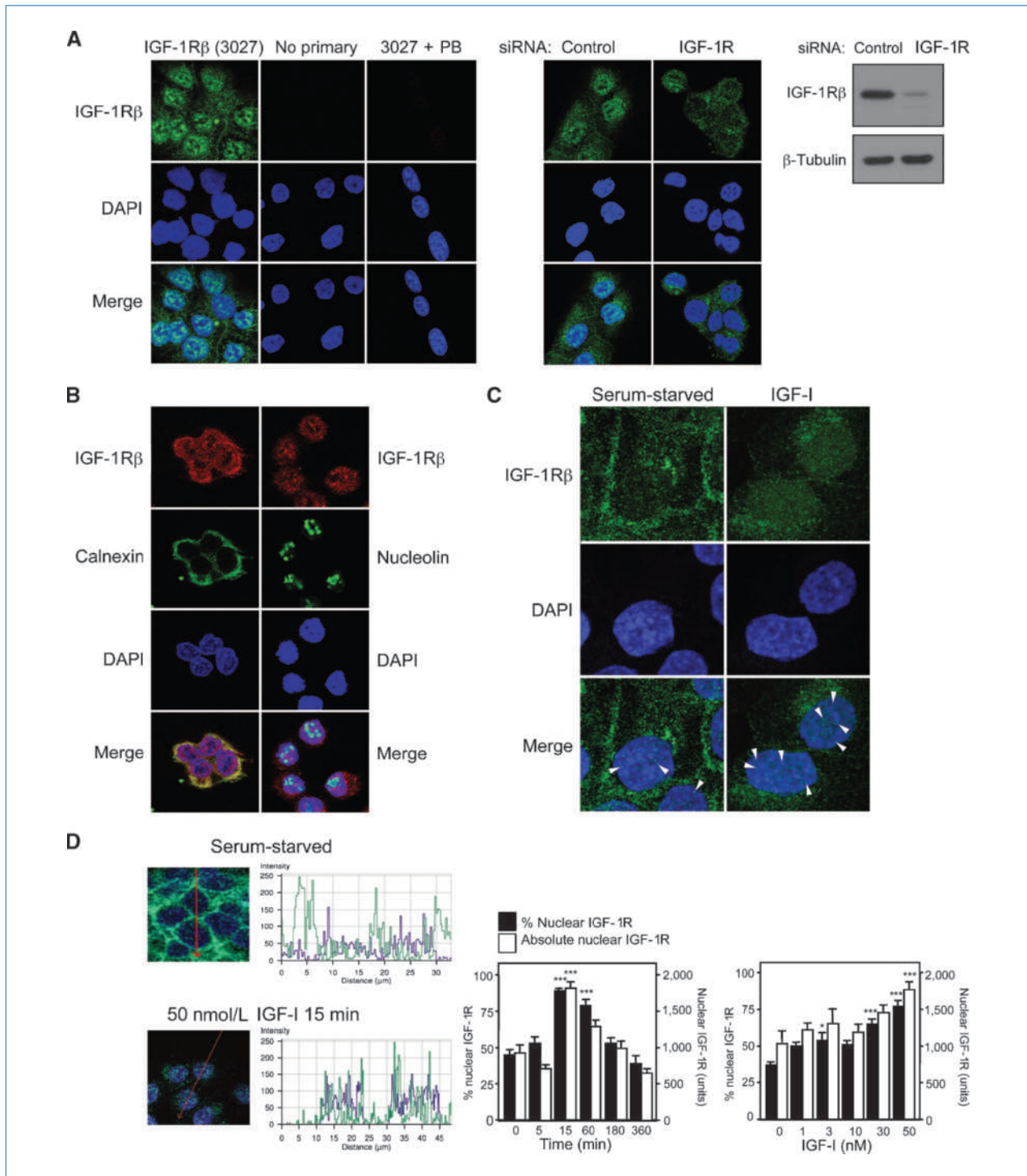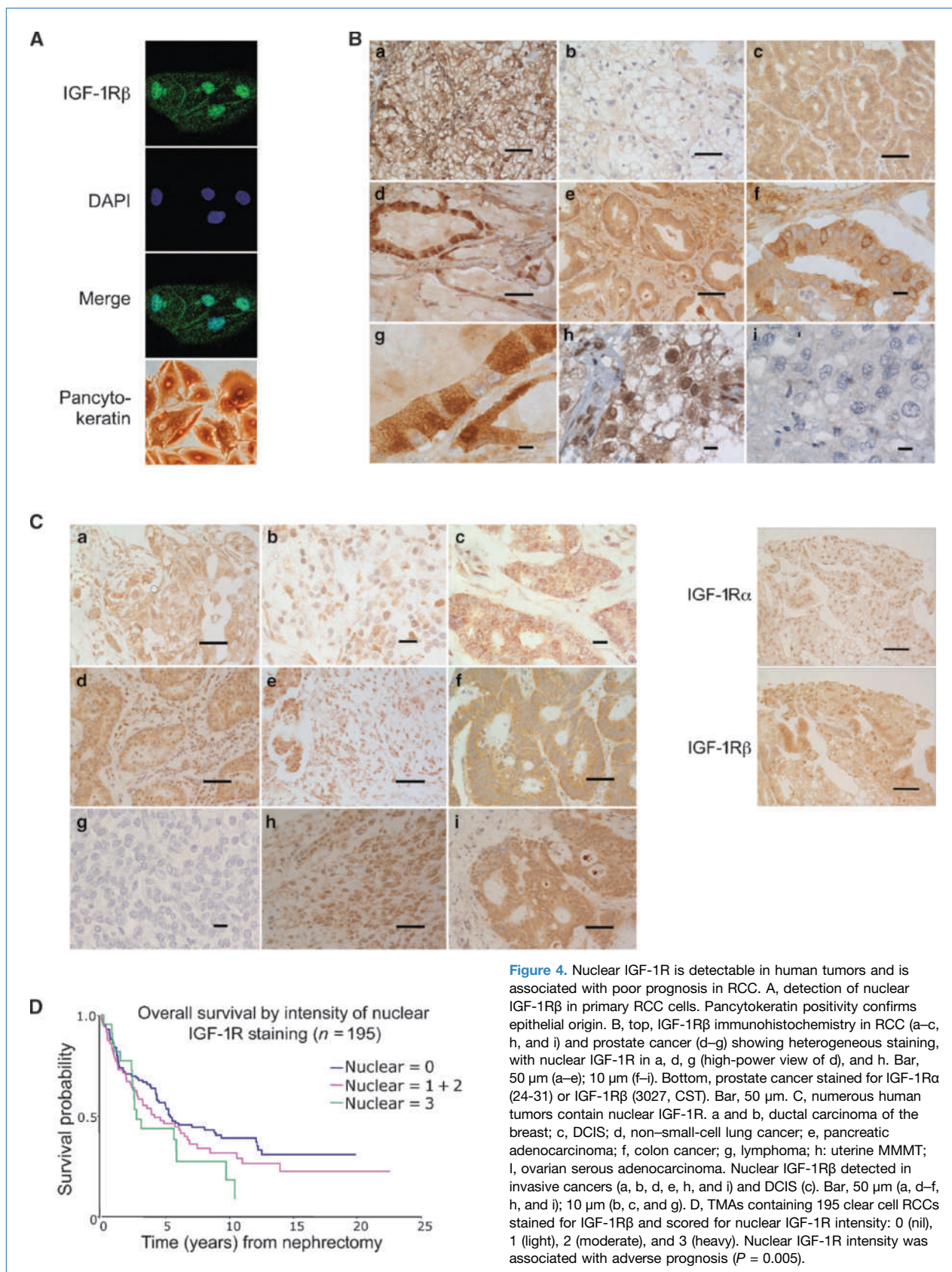
Figure 1. IGF-I induces IGF-1R nuclear translocation in human tumor cells. A, IGF-1Rβ immunofluorescence in DU145 cells cultured in complete medium. IGF-1R signal was attenuated by IGF-1R depletion (confirmed in immunoblot to right). B, DU145 cells co-stained for IGF-1R and calnexin or nucleolin. C, serum-starved DU145 cells were treated with solvent or IGF-I (50 nmol/L, 15 min) and stained for IGF-1Rβ as in A. Arrowheads, examples of punctate nuclear IGF-1R. Original magnification, ×100. D, left, DU145 cells were serum starved or IGF treated (50 nmol/L, 15 min) and stained for IGF-1Rβ and 4′,6-diamidino-2-phenylindole (DAPI). Merged images; arrow shows path along which the intensity of IGF-1R (green) and DAPI (blue) is quantified. Center, IGF-1R overlying DAPI registers ~50 arbitrary units in starved cells (top) and 100 to 150 units after IGF-I treatment (bottom). Right, quantification of nuclear IGF-1R after treatment with 50 nmol/L IGF-I for 0 to 360 min (left) and 0 to 50 nmol/L IGF-I for 15 min (right). Black columns, mean percent nuclear IGF-1R; white columns, mean absolute nuclear IGF-1R (arbitrary units); bars, SEM (n = 20–30 cells). Compared with serum-starved cells, nuclear IGF-1R signal was enhanced by IGF-I (*, $P < 0.05$; ***, $P < 0.001$).
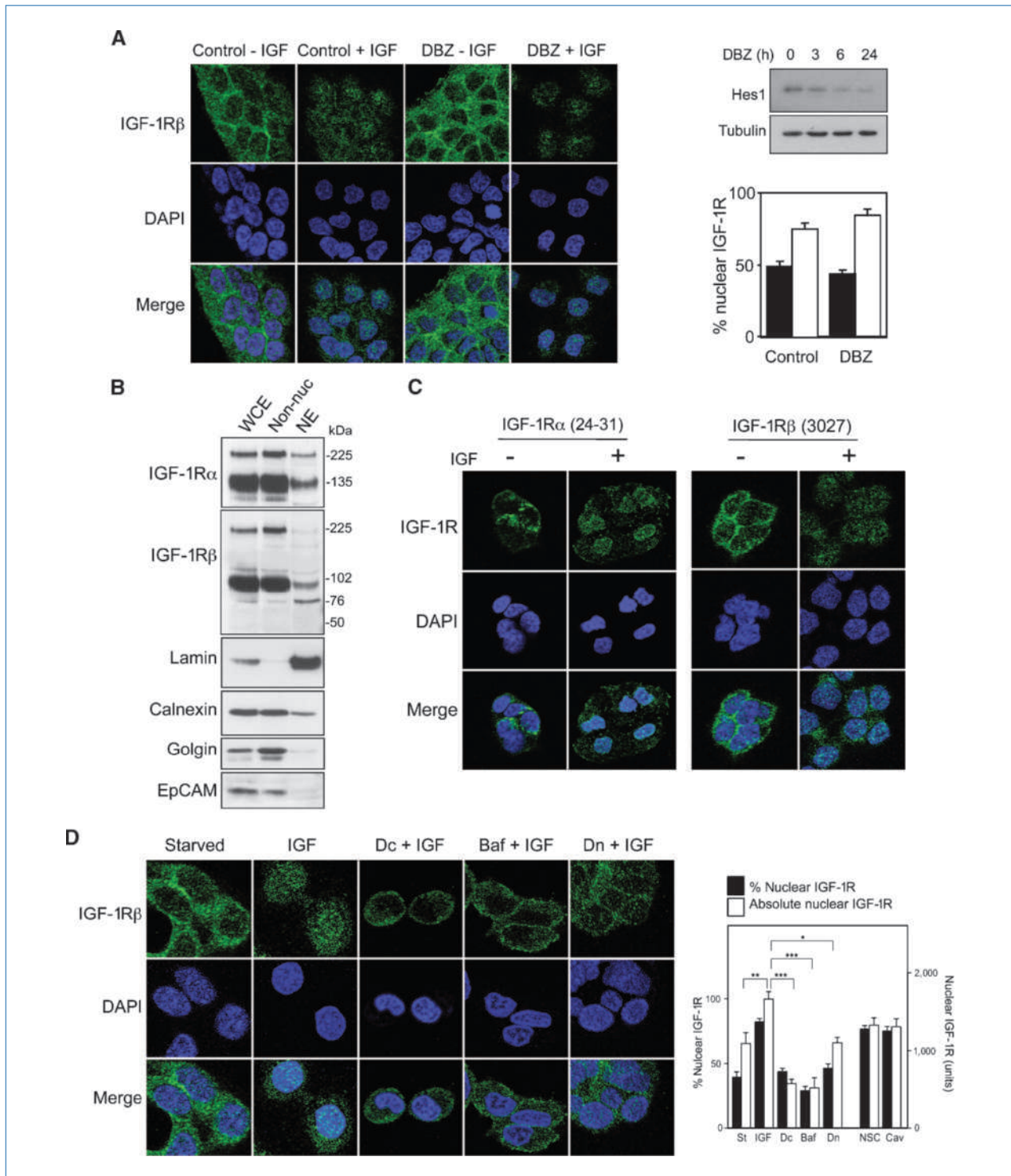
Figure 2. Full-length IGF-1R α and β subunits undergo nuclear import following clathrin-dependent endocytosis. A, DU145 cells were treated with dibenzazepine (DBZ; 300 nmol/L, 6 h) and in the final 15 min with 50 nmol/L IGF-I. Graph: mean percent nuclear IGF-1R in serum-starved (black columns) or IGF-treated cells (white columns); bars, SEM. Immunoblotting (top right) confirmed DBZ bioactivity in inhibiting expression of the Notch target Hes1. B, DU145 whole-cell extract (WCE), non-nuclear components (Non-nuc), and nuclear extract (NE) immunoblotted for IGF-1R, lamin (nucleus), calnexin (ER), golgin-84 (Golgi), and EpCAM (plasma membrane). C, serum-starved DU145 cells were treated with solvent or IGF-I (50 nmol/L, 15 min) and stained for IGF-1Rα or IGF-1Rβ. D, serum-starved DU145 cells were treated for 4 h with dansylcadaverine (Dc), bafilomycin A1 (Baf), or dynasore (Dn) and in the final 15 min with 50 nmol/L IGF-I. Absolute nuclear IGF-1R was enhanced by IGF-I (**, $P < 0.01$) and inhibited by Dc, Baf, and Dn (*, $P < 0.05$; ***, $P < 0.001$). Supplementary Fig. S5A shows images of IGF-treated cells following caveolin-1 depletion.
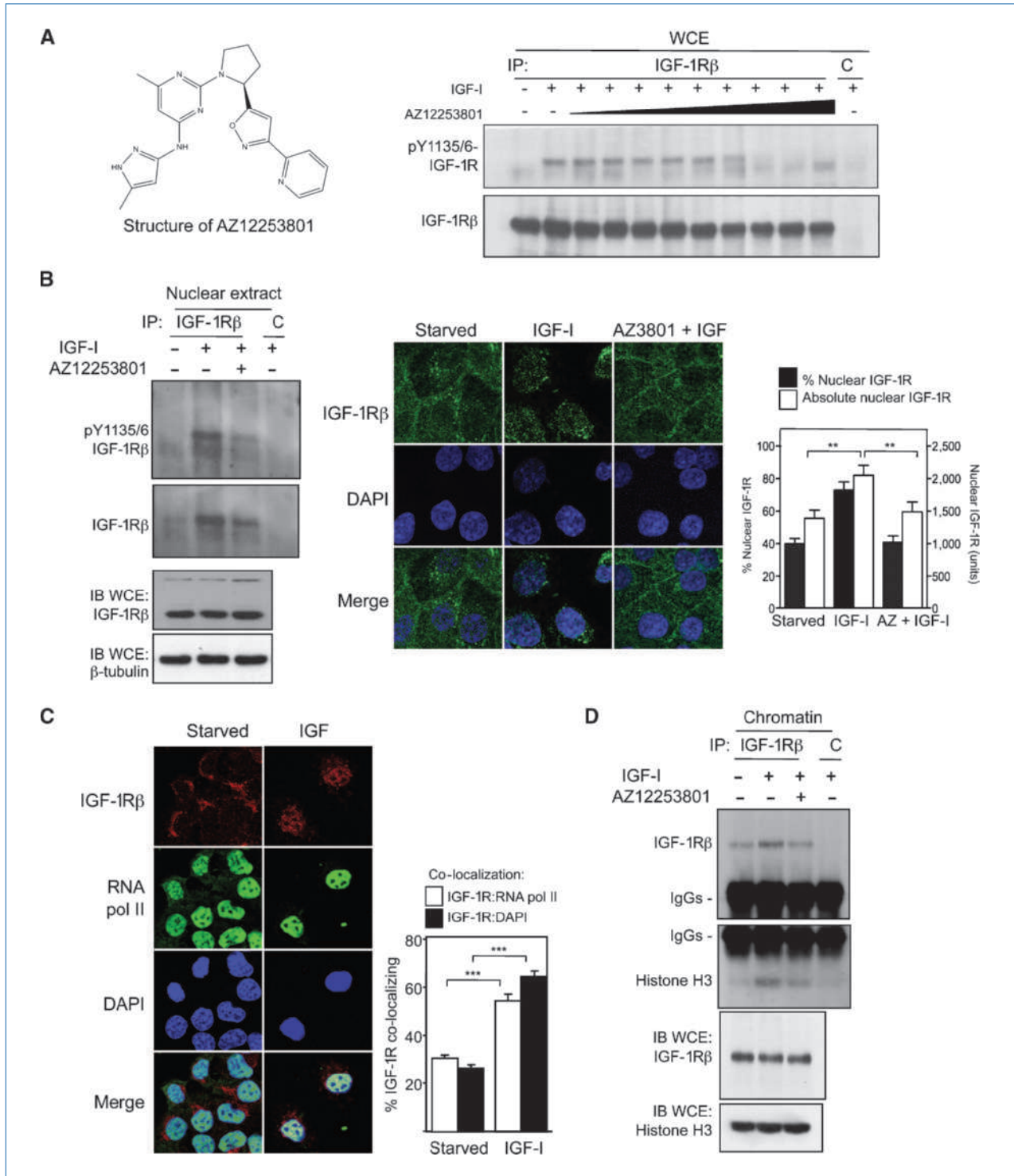
Figure 3. IGF-1R nuclear import and chromatin binding are blocked by IGF-1R inhibition. A, left, structure of AZ12253801; right, serum-starved DU145 cells treated with 50 nmol/L IGF-I in the final 15 min of 1-h incubation with 0.1 to 100 nmol/L AZ12253801. IGF-1Rβ or control (C) immunoprecipitates probed for phospho- and total IGF-1Rβ. Supplementary Fig. S6 shows quantification of these results and effects on clonogenic survival. B, DU145 cells treated with 50 nmol/L IGF-I in the final 30 min of 6-h incubation with 120 nmol/L AZ12253801. Left, nuclear extracts immunoprecipitated with control (C) or IGF-1Rβ antibody and probed for phospho- and total IGF-1Rβ. Right, parallel cultures imaged for IGF-1Rβ. IGF-induced nuclear IGF-1R import was inhibited by AZ12253801 ($P < 0.001$, for percent nuclear signal; **, $P < 0.01$, for absolute nuclear signal). C, serum-starved and IGF-treated cells were co-stained for IGF-1Rβ (red) and RNA polymerase II (green). IGF-I enhanced co-localization of IGF-1R with RNA pol II and DAPI (***, $P < 0.001$). D, after treatment with AZ12253801 and IGF-I as B), IGF-1Rβ was immunoprecipitated from chromatin and probed for IGF-1Rβ and histone H3.

Figure 4. Nuclear IGF-1R is detectable in human tumors and is associated with poor prognosis in RCC. A, detection of nuclear IGF-1Rβ in primary RCC cells. Pancytokeratin positivity confirms epithelial origin. B, top, IGF-1Rβ immunohistochemistry in RCC (a–c, h, and i) and prostate cancer (d–g) showing heterogeneous staining, with nuclear IGF-1R in a, d, g (high-power view of d), and h. Bar, 50 μm (a–e); 10 μm (f–i). Bottom, prostate cancer stained for IGF-1Rα (24-31) or IGF-1Rβ (3027, CST). Bar, 50 μm. C, numerous human tumors contain nuclear IGF-1R. a and b, ductal carcinoma of the breast; c, DCIS; d, non–small-cell lung cancer; e, pancreatic adenocarcinoma; f, colon cancer; g, lymphoma; h: uterine MMMT; l, ovarian serous adenocarcinoma. Nuclear IGF-1Rβ detected in invasive cancers (a, b, d, e, h, and i) and DCIS (c). Bar, 50 μm (a, d–f, h, and i); 10 μm (b, c, and g). D, TMAs containing 195 clear cell RCCs stained for IGF-1Rβ and scored for nuclear IGF-1R intensity: 0 (nil), 1 (light), 2 (moderate), and 3 (heavy). Nuclear IGF-1R intensity was associated with adverse prognosis ($P = 0.005$).

(Supplementary Figs. S3 and S6). Therefore, we interrogated the contribution of the IGF-1R kinase to nuclear translocation, using two classes of IGF-1R inhibitor: the blocking antibody MAB391 and a selective inhibitor of the IGF-1R kinase, AZ12253801 (Fig. 3A; Supplementary Fig. S6). Equimolar concentrations of each agent inhibited IGF-1R activation, and consistent with previous findings (21), MAB391 downregulated whole-cell IGF-1R. In whole-cell extracts and in the nuclear compartment, IGF-1R phosphorylation was more profoundly inhibited by AZ12253801 than by MAB391 (Supplementary Fig. S6C). Pretreatment with AZ12253801 at its IC_{50} for proliferation not only blocked nuclear IGF-1R phosphorylation but also inhibited IGF-1R nuclear import, shown by immunoprecipitation from nuclear extract and confocal microscopy (Fig. 3B). These data indicate that IGF-1R kinase activity is required for IGF-1R to enter the nucleus. This information may have therapeutic relevance: aside from the challenge of crossing membranes, antibodies may be limited by their size from entering the nucleus in complex with IGF-1R (22); we speculate that nuclear IGF-1R activity may be more effectively blocked by small-molecule inhibitors. In considering potential functions of the receptor in this newly identified location, we observed that the distribution of nuclear IGF-1R was reminiscent of the speckled pattern characteristic of components of the transcriptional machinery (23). Furthermore, we noted (Fig. 2C) that punctate nuclear IGF-1R was located principally in less dense regions of DNA, which are potentially more accessible to transcription factors. Indeed, we could detect IGF-induced co-localization with RNA polymerase II (Fig. 3C), and binding to chromatin was shown by coprecipitation with histone H3 (Fig. 3D). This suggests a direct role for the IGF-1R in transcriptional regulation, consistent with the recent report of Sehat and colleagues (20) and with the known function of other nuclear RTKs (5, 7).

Finally, we investigated the clinical relevance of these findings. Nuclear IGF-1R was evident in primary RCC cultures (Fig. 4A) and formalin-fixed RCC and prostate cancers, with marked heterogeneity between and within tumors (Fig. 4B). Consistent with detection of both IGF-1R subunits in the nuclei of cultured tumor cells (Fig. 2B and C), prostate cancers contained nuclear α- and β-immunoreactivity (Fig. 4B, bottom). Nuclear IGF-1R was detectable in additional tumor types including adenocarcinomas of the breast, lung, and ovary and ductal carcinoma *in situ* (DCIS; Fig. 4C; Supplementary Table S1). The IGF-1R seemed almost exclusively nuclear in some breast and pancreatic cancers and malignant mixed Müllerian tumor, a rare, aggressive uterine malignancy (Fig. 4C). Nuclear IGF-1R was also observed in benign epithelia of the esophagus, lung, breast, cervix, and prostate and in germ cells in the testis (Supplementary Table S1; Supplementary Fig. S7). This pattern suggests an association with proliferation, as reported for nuclear EGFR (5). Finally, we analyzed a series of clear cell RCCs, in which total IGF-1R expression is reported to have prognostic significance (24). Nuclear IGF-1R was detectable in 94 of 195 (48%) of clear cell RCCs. Multivariate analysis identified known prognostic factors (age, tumor grade, stage) and revealed that survival was shorter in patients whose tumors showed intense ($P = 0.005$) and/or widespread ($P = 0.003$) nuclear IGF-1R (Fig. 4D; Supplementary Table S2).

In conclusion, these findings support the concept that nuclear IGF-1R has biological significance. These data provide new insights into IGF biology and may have implications for use of IGF-1R inhibitors in cancer therapy.

Disclosure of Potential Conflicts of Interest

Acknowledgments

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