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The c-Yes tyrosine kinase is a potent suppressor of ES cell differentiation and antagonizes the actions of its closest phylogenetic relative, c-Src

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Abstract

ES cells are derived from the inner cell mass of the blastocyst stage embryo and are characterized by self-renewal and pluripotency. Previous work has shown that Src-family tyrosine kinases display dynamic expression and activity changes during ES cell differentiation, suggesting distinct functions in the control of developmental fate. Here we used ES cells to test the hypothesis that c-Src and its closest phylogenetic relative, c-Yes, act in biological opposition despite their strong homology. Unlike c-Src, enforced expression of active c-Yes blocked ES cell differentiation to embryoid bodies by maintaining pluripotency gene expression. To explore the interplay of c-Src and c-Yes in ES cell differentiation, we engineered c-Src and c-Yes mutants that are resistant to A-419259, a potent pyrrolopyrimidine inhibitor of the Src kinase family. Previous studies have shown that A-419259 treatment blocks all Src-family kinase activity in ES cells, preventing differentiation while maintaining pluripotency. Expression of inhibitor-resistant c-Src but not c-Yes rescued the A-419259 differentiation block, resulting in a cell population with properties of both primitive ectoderm and endoderm. Remarkably, when inhibitor-resistant c-Src and c-Yes were expressed together in ES cells, c-Yes activity suppressed c-Src mediated differentiation. These studies show that even closely related kinases such as c-Src and c-Yes have unique and opposing functions in the same cell type. Selective agonists or inhibitors of c-Src vs. c-Yes activity may allow more precise pharmacological manipulation of ES cell fate and have broader applications in other biological systems which express multiple Src family members such as tumor cells.

Keywords

Src-family kinases; c-Yes kinase; ES cells; chemical genetics

INTRODUCTION

Embryonic stem (ES) cells are derived from the inner cell mass of the developing blastocyst[1, 2]. ES cells are characterized by self-renewal, the ability to multiply indefinitely without differentiation[3], and pluripotency, the developmental potential to generate cell types from all three germ layers[4, 5]. In the absence of feeder cell layers, ES cells can be maintained in an undifferentiated state by culturing them in serum-based medium supplemented with the cytokine, leukemia inhibitory factor (LIF)[6] or in defined medium in the presence of LIF and bone morphogenetic proteins (BMPs)[7]. In the absence of LIF, ES

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cells differentiate to embryoid bodies (EBs) when cultured under non-adherent conditions. EB formation mimics the earliest stages of embryonic development, giving rise to all three germ layers[8, 9].

Multiple intracellular kinase signaling pathways play a dominant role in the regulation of ES cell fate[10, 11], with at least four pathways important for self-renewal. LIF signals through Janus kinases (Jaks) and signal transducer and activator of transcription 3 (STAT3). This pathway promotes expression of renewal factors including the POU domain transcription factor, Oct4[12], and the homeobox transcription factor, Nanog[13, 14]. Bone morphogenetic proteins (BMPs), which are serum components, activate transcription factors of the SMAD family and inhibit differentiation through induction of inhibitor of differentiation (ID) factors[7]. Wnt proteins, which are also found in serum, inhibit glycogen synthase kinase-3β activity, leading to β-Catenin accumulation and pluripotency marker gene expression[15, 16]. In addition, the phosphatidylinositol 3'-kinase (PI3K) signaling pathway promotes ES cell self-renewal partly via regulation of Nanog expression[17, 18].

Previous work has implicated the Src family of non-receptor protein tyrosine kinases in self-renewal and differentiation of murine ES cells as well[19, 20]. Seven of the eight mammalian Src family members are expressed in murine ES cells, and several family members are active in cycling ES cells cultured in the presence of LIF and serum (c-Src, c-Yes, Fyn, and Hck). Accumulating evidence supports the hypothesis that individual members of this kinase family may play distinct roles in regulating ES cell fate. For example, early studies showed that expression of an active mutant of Hck reduces the LIF requirement for ES cell self-renewal, implicating Hck in the suppression of differentiation[21]. More recent studies from our group showed that transcription of Hck is rapidly silenced as ES cells differentiate to EBs, consistent with this idea[20]. In contrast to Hck, active c-Src is expressed in both ES cells and differentiated EBs. Moreover, when c-Src remains active in the absence of all other Src-family kinase activity, it is sufficient to induce differentiation of ES cells[22].

Other work has linked c-Yes, the closest phylogenetic relative of c-Src, to the suppression of ES cell differentiation. Like c-Src, c-Yes is expressed in both pluripotent ES cells and in differentiated EBs[19]. While the c-Yes kinase is active in self-renewing ES cells, where it is regulated by both LIF and serum, its activity is downregulated during differentiation. RNAi-mediated knockdown of c-Yes function reduces expression of the renewal factor Nanog while increasing expression of the differentiation marker, GCNF. Transcription of c-Yes in ES cells is regulated by the pluripotency factor Oct4, supporting a role for c-Yes in self-renewal[23]. Recent work shows that active c-Yes controls the TEAD2 transcription factor through the Yes-associated protein, YAP[24]. Active YAP-TEAD2 complexes bind Oct4 promoters, supporting a positive feedback loop between c-Yes and Oct4 in self-renewal.

In this study we examined the biological interplay of c-Yes and c-Src, closely homologous kinases independently shown to produce opposite biological outcomes in ES cells. First, we expressed c-Yes in mouse ES cells using a retroviral vector system that drives low-level protein expression in transduced ES cell populations[22]. EB maturation was completely blocked in ES cells expressing active c-Yes, while EBs formed by ES cells expressing a kinase-inactive c-Yes mutant were unaffected. EBs that formed from the c-Yes-transduced ES cell population expressed both pluripotency and differentiation markers, suggesting that c-Yes kinase activity prevents differentiation by maintaining expression of the self-renewal program. Using a chemical genetics approach that permits only c-Yes and c-Src signaling in ES cells in the absence of all other SFK signaling, we found that c-Yes interfered with the induction of differentiation previously observed with c-Src in this system[22]. In addition, we found that c-Yes also suppressed the induction of the epithelial-mesenchymal transition (EMT) by c-Src. Together, these observations show that despite their high degree of

sequence similarity, c-Yes and c-Src have opposing roles in the regulation of ES cell fate. Our results have important implications not only for the regulation of ES cell differentiation, but also for the many other cellular contexts in which multiple members of this kinase family are expressed.

RESULTS

Downregulation of c-Yes kinase activity during differentiation of ES cells to EBs

To monitor changes in c-Yes expression and activity during ES cell self-renewal and differentiation, we compared endogenous c-Yes RNA levels, protein abundance and kinase activity in self-renewing ES cells vs. EBs following 6 days in differentiation culture (6-day EBs). For these experiments, we used the mouse ES cell line D3, which readily forms EBs when plated under non-adherent conditions in the absence of LIF (Figure S1A)[20]. Total RNA was isolated from self-renewing ES cells and 6-day EBs, followed by quantitative real-time RT-PCR (qPCR) analysis of c-Yes and c-Src transcript levels as well as markers of self-renewal and differentiation. Differentiation to EBs resulted in negligible changes in the expression of c-Yes and c-Src (Figure S1B). Expression of the self-renewal markers Oct4 and Nanog were significantly down-regulated while the lineage-specific differentiation markers Gata4 (endoderm), Fgf5 (ectoderm) and T (mesoderm) were up-regulated in the 6-day EBs, consistent with the onset of differentiation.

To investigate c-Yes protein stability and activity as a function of differentiation, c-Yes was immunoprecipitated from ES cells and 6-day EBs, followed by immunoblotting with a c-Yes phosphospecific antibody. As shown in Figure S1C, activation loop tyrosine phosphorylation was greatly diminished in EBs relative to ES cells despite equivalent recovery of c-Yes protein. Immunoblots also showed a dramatic reduction in the level of the pluripotency marker, Oct4, consistent with differentiation. This result shows that c-Yes kinase activity is downregulated during EB formation, consistent with a role for this Src-family member in maintenance of pluripotency as originally proposed by Anneren et al.[19].

Validation of c-Yes retroviral expression constructs in Rat2 cells

Results presented in the previous section suggest that downregulation of c-Yes kinase activity may be required for ES cells to exit the self-renewal program. To test this hypothesis, we engineered a series of c-Yes retroviral expression constructs based on a murine stem cell virus (MSCV) promoter which we have previously shown to remain active in both self-renewing ES cells and differentiated EBs[22]. In addition, the c-Yes cDNA clones were coupled to a G418 selection marker via an internal ribosome entry site (IRES) to ensure stable expression in the transduced cell populations.

Like all Src kinases, c-Yes consists of an N-terminal unique region, followed by SH3 and SH2 domains, the kinase domain, and a negative regulatory tail[25]. To create an inactive mutant of c-Yes, we substituted Lys295 in the kinase domain with Arg. In addition, we created a constitutively active form of c-Yes by replacement of the negative regulatory tail tyrosine (Tyr527) with phenylalanine (Figure S2A). To validate the c-Yes retroviral expression vectors, we used Rat2 fibroblasts as a model system. Rat2 cells were transduced with the wild-type, kinase-dead and kinase-active forms of c-Yes, followed by soft agar colony assays for anchorage independent growth. As shown in Figures S2B and S2C, cells expressing the active mutant of c-Yes produced a large number of transformed colonies, while no colonies were observed with cells expressing wild-type c-Yes or the kinase-dead mutant. Expression of c-Yes from the retroviral vector was confirmed by RT-PCR using a primer pair specific for the c-Yes transgene (Figure S2D).

To assay for c-Yes kinase activity in each of the Rat2 cell populations, c-Yes was immunoprecipitated followed by immunoblotting for protein recovery and activation loop tyrosine phosphorylation. Figure S2E shows a small increase in the quantity of c-Yes protein recovered from cells expressing the c-Yes cDNAs relative to the vector controls. The tail mutant of c-Yes reacted strongly with the phosphospecific antibody, consistent with elevated kinase activity and transforming function. In contrast, c-Yes recovered from cells expressing the wild-type protein showed only a small increase in reactivity with the activation loop antibody relative to endogenous c-Yes. This result demonstrates that the level of wild-type c-Yes over-expression achieved with the MSCV-based retrovirus was not sufficient to cause kinase upregulation, consistent with the lack of transformed colony formation.

Low-level retroviral expression of c-Yes in ES cells does not affect undifferentiated colony morphology or marker expression

To test the effect of these c-Yes constructs on self-renewal, cultures of ES cells were transduced with recombinant retroviruses carrying the three forms of c-Yes described above. Following selection with G418, each ES cell population formed undifferentiated colonies indistinguishable from control cells transduced with the empty vector (Figure S3A). RT-PCR with a primer pair specific for the retroviral transgene showed that c-Yes is expressed in all three cell populations transduced with c-Yes but not in the vector control cells (Figure S3B). The presence of the kinase domain mutations was confirmed in the RT-PCR products from the transduced cell lines by DNA sequence analysis (data not shown). The relative growth rate of each c-Yes-transduced ES cell populations was indistinguishable from that of control ES cells (data not shown). Finally, qPCR analysis showed that the expression levels of the pluripotency markers Rex1, Nanog and Oct4, as well as the differentiation markers Fgf5 (ectoderm) and T (mesoderm) were essentially unchanged in each of the c-Yes-transduced ES cell populations relative to control ES cells (Figure S3C). These results indicate that retroviral transduction with c-Yes expression vectors did not overtly affect the regulation of self-renewal in cycling mouse ES cells.

ES cells expressing active c-Yes fail to form EBs

Previous studies support a role for the c-Yes kinase in the maintenance of mES cell self-renewal[19, 24]. To determine whether c-Yes kinase activity must be downregulated for differentiation to proceed, ES cells expressing all three forms of c-Yes were cultured without LIF to induce EB formation. As shown in Figure 1, control ES cells and cells expressing kinase-dead c-Yes formed spherical clusters after 6 days, consistent with normal differentiation to EBs. In contrast, ES cells expressing either wild-type c-Yes or the kinase-active mutant formed much smaller irregularly shaped groups of cells. Size analysis revealed that the EBs derived from ES cells expressing the wild-type or active forms of c-Yes were significantly smaller than those derived from control ES cells or cells expressing kinase-dead c-Yes (Figure 1). Remarkably, the small increase in c-Yes activity expected to result from expression of wild-type c-Yes (Figure S2E) is sufficient to interrupt normal EB development. This suggests that endogenous c-Yes activity must be tightly regulated to ensure normal ES cell differentiation to EBs.

ES cells expressing active c-Yes kinases express both pluripotency and differentiation markers during EB formation

ES cells transduced with active c-Yes were significantly impaired in their ability to form EBs of similar size to those derived from control cells. To investigate the mechanism of this differentiation defect, pluripotency and differentiation marker expression profiles were compared in 6-day EBs formed from each of the undifferentiated ES cell populations. As shown in Figure 2A, levels of the pluripotency markers Rex1, Dppa4, Esrrb, Klf2, Klf4,

Oct4 and Nanog were significantly higher in EBs derived from ES cells expressing either wild-type or the active mutant of c-Yes compared with those from control ES cells or from cells expressing kinase-dead c-Yes. These results link c-Yes kinase activity to the expression of genes directly involved in self-renewal. The failure of these cells to downregulate pluripotency gene expression may account for their failure to form EBs. Similar results were seen with cells expressing either the wild-type or the constitutively active c-Yes kinases, providing further evidence that even a small increase in c-Yes kinase activity is sufficient to disrupt mES cell differentiation.

We also explored changes in differentiation marker expression in the EBs derived from the same four ES cell populations. As shown in Figure 2B, Sox17/Gata4 (endoderm), Pax6, Fgf5 (ectoderm), Fgf8 (epiblast) and T (mesoderm) were expressed at similar levels across all four cell populations following six days of EB culture conditions. This observation suggests that c-Yes kinase activity does not interfere with differentiation marker gene expression.

Design of c-Yes gatekeeper mutants resistant to the broad spectrum Src-family kinase inhibitor, A-419259

Selective kinase inhibitors represent valuable probes for biological function. However, isoform-selective inhibitors of c-Yes or other members of the Src-kinase family are currently unavailable, due to the close sequence and structural similarity of the individual family members. To circumvent this issue, we turned to a chemical genetics approach previously developed in our laboratory to demonstrate a role for c-Src kinase activity in ES cell differentiation[22]. This method paired the broad-spectrum Src-family kinase inhibitor A-419259 with a c-Src variant engineered to be resistant to this compound. Introduction of the inhibitor-resistant (Src-IR) mutant into ES cells had no effect in the absence of inhibitor treatment. However, ES cells expressing Src-IR differentiated upon addition of A-419259, indicating that c-Src kinase activity alone is sufficient to induce this response.

To create analogous IR variants of c-Yes, we substituted the threonine residue at the c-Yes kinase domain gatekeeper position (T338) with methionine (Figure S4A) in the context of the tail-activated (Y527F) form of c-Yes described above. Tail-activated mutants of c-Yes with wild-type and IR kinase domains were then expressed in Rat2 cells, followed by soft-agar colony assays in the presence of A-419259. Figure S4B shows that colony formation by Rat2 cells expressing tail-activated c-Yes with a wild-type kinase domain was very sensitive to A-419259 treatment, while colony formation by cells expressing tail-activated c-Yes-IR was unaffected. Immunoblot analysis showed that c-Yes from cells transformed by active Yes with a wild-type kinase domain was inhibited by A-419259 treatment, while c-Yes-IR remained active at all concentrations of A-419259 tested (Figure S4C). These experiments show that methionine substitution of the c-Yes gatekeeper threonine results in inhibitor resistance as observed previously with c-Src.

Stable expression of inhibitor-resistant c-Yes (Yes-IR) in ES cells does not affect self-renewal marker expression

We next expressed the wild-type or IR forms of c-Yes in ES cells. In the absence of A-419259 treatment, these ES cell populations formed colonies indistinguishable from control ES cells (Figure 3A). In the presence of A-419259, cells expressing wild-type c-Yes as well as the vector control cells formed small tight colonies, consistent with our previous results with ES cells grown in the presence of this inhibitor[20, 22]. In contrast, the Yes-IR ES cells formed larger colonies compared to control ES cells, with some flattened colony morphology, in the presence of the inhibitor. Active c-Yes was readily detected in Yes-IR expressing cells treated with A-419259, but not in control cells or cells expressing wild-type

c-Yes, verifying that Yes-IR remains active in A-419259 treated cells (Figure 3B). No significant expression changes were observed following qPCR analysis of the pluripotency markers Rex1, Nanog and Oct4 or the differentiation markers T (mesoderm) and Fgf5 (ectoderm) in either c-Yes cell population. These experiments suggest that under conditions where c-Yes is the lone active Src family member present, no major changes in ES cell pluripotency or differentiation occur.

Differentiation of ES cells driven by c-Src is antagonized by c-Yes

Previous work from our group demonstrated that c-Src activity alone is sufficient to drive differentiation of ES cells using an analogous inhibitor-resistant allele of c-Src and A-419259[22]. In contrast, work presented here demonstrates that despite close structural homology to c-Src, c-Yes activity inhibits rather than promotes ES cell differentiation. To determine which of these two opposing signals dominates, we introduced the IR forms of both c-Yes and c-Src into ES cells. When cultured in the absence of A-419259, the resulting cell populations grew with similar undifferentiated colony morphology (Figure 4A). Ectopic expression of the c-Yes and c-Src transgenes was confirmed by RT-PCR with vector-specific primers (Figure 4B). No changes in pluripotency or differentiation marker expression were observed in cells expressing c-Src-IR, c-Yes-IR or both IR mutants in the absence of A-419259 treatment (Figure 4C).

We next examined the effect of A-419259 treatment on cell morphology as well as pluripotency and differentiation marker gene expression in ES cells expressing Src-IR or Yes-IR either alone or in combination. Following 4 days of A-419259 treatment in the presence of LIF, control ES cells formed tight, small colonies, while colonies formed from the c-Yes-IR cells showed a more flattened morphology as described earlier. In contrast, cells expressing c-Src-IR or both inhibitor-resistant alleles grew as flat sheets in the presence of the inhibitor (Figure 5A). RT-PCR showed that inhibitor treatment did not result in the silencing of c-Src-IR or c-Yes-IR transgene expression (Figure 5B). We then assayed expression of pluripotency markers (Tbx3, Rex1, Nanog, Oct4 and Gbx2; Figure 5C) as well as differentiation markers for primitive endoderm (Sox17, Gata4, Gata6), ectoderm (Fgf5, Sox1, Nestin), mesoderm (T), epiblast (Fgf8) and trophectoderm (Hand1) by qPCR relative to the control ES cell population (Figure 5D). Inhibitor treatment of ES cells expressing c-Src-IR alone resulted in the upregulation of Gbx2, Sox17, Gata4, Gata6, Fgf5 and Hand1 expression, indicative of differentiation towards endoderm and trophectoderm in addition to primitive ectoderm-like cells as reported previously by our group[22]. This finding suggests that c-Src regulates a much broader range of differentiation responses than originally reported. In contrast to c-Src, the c-Yes-IR cells did not express any of these differentiation markers in response to A-419259 treatment. This observation is consistent with the idea that c-Yes signals help to maintain ES cells in an undifferentiated state.

Expression of both resistant alleles in the same cell population revealed dominance of c-Yes over c-Src in terms of a subset of differentiation marker expression. As shown in Figure 5D, induction of the endoderm markers Sox17, Gata4 and Gata6 by c-Src was significantly repressed in cells expressing IR alleles of both c-Src and c-Yes vs. c-Src alone. In contrast, expression of Gbx2, Fgf5 and Hand1 remained the same when both IR alleles were expressed, raising the possibility that other renewal-related Src-family kinases (e.g., Hck) may influence these pathways. Changes in primitive endodermal marker gene expression (Gata6) across the four ES cell populations were confirmed at the single cell level by immunofluorescence microscopy (Figure S5). Cells that did not stain positive for Gata6 are likely to have differentiated along other lineages (e.g., primitive ectoderm or trophectoderm).

Recent studies have shown that c-Src promotes the EMT[26], a process essential for lineage specification during development[27]. To investigate a possible connection of the EMT to Src-induced differentiation of ES cells, we assayed the relative expression levels of EMT markers previously linked to c-Src, including Igf2, SIP1, Ncad, Snail1, Twist1 and Eomes as well as matrix metalloproteinases (MMPs). As shown in Figure 5E, A-419259 treatment of ES cells expressing c-Src-IR alone resulted in up-regulation of these EMT markers (with the exceptions of SIP1 and Eomes), consistent with a role of the EMT in c-Src-mediated differentiation of ES cells. In contrast, c-Yes-IR cells did not upregulate any of these EMT markers following A-419259 treatment. Interestingly, co-expression of c-Yes-IR suppressed c-Src-IR-mediated induction of Snail1, Twist1 and MMP14 expression, with a partial reversal of MMP9 expression upon inhibitor treatment. The opposing roles of c-Src vs. c-Yes in the EMT across the four ES cell populations were confirmed at the single cell level by immunofluorescence microscopy for the additional EMT marker, E-Cadherin (Figure S6). Taken together, these results show that c-Src activity alone is sufficient to promote EMT marker expression and differentiation, while c-Yes activity inhibits both c-Src mediated EMT and endodermal differentiation.

DISCUSSION

Work presented here shows that the Src-family kinase c-Yes generates a potent anti-differentiation signal in mouse ES cells. Enforced expression of either wild-type or an active form of c-Yes at modest levels completely inhibited differentiation of ES cells to EBs. This suppressive effect was not observed with kinase-defective c-Yes, indicating a requirement for c-Yes kinase activity. Interestingly, expression of both wild-type c-Yes as well as a kinase-active ‘tail’ mutant inhibited EB formation to the same extent, showing that ES cells are very sensitive to the c-Yes anti-differentiation signal. Our observations are consistent with prior studies showing that c-Yes kinase activity is stimulated by the self-renewal cytokine LIF and that RNAi-mediated knockdown of c-Yes induces ES cell differentiation[19]. Although c-Yes kinase activity interferes with EB formation, it cannot sustain self-renewal following LIF withdrawal. Indeed, ES cells expressing the active forms of c-Yes undergo morphological differentiation when cultured under adherent conditions in the absence of LIF (data not shown). Furthermore, qPCR analysis shows that the small EBs formed from ES/c-Yes cells continue to express pluripotency factors, including Oct4 and Nanog, despite the onset of differentiation marker expression (Figure 2). Thus the presence of active c-Yes appears to prevent EB formation by interfering with the repression of pluripotency genes as opposed to blocking the differentiation program.

Seven of the eight mammalian Src family members are expressed simultaneously in self-renewing mouse ES cells[20], making investigation of their individual contributions to self-renewal and differentiation a challenge. To address this problem, we applied a chemical genetics approach based on the broad-spectrum Src-family kinase inhibitor, A-419259. Treatment of ES cells with this inhibitor blocks all endogenous Src-family kinase activity in ES cells, locking them in an undifferentiated state[20]. Expression of a c-Src mutant with engineered resistance to this inhibitor caused the ES cells to differentiate to primitive ectoderm-like cells[22]. This chemical genetics approach allowed us to demonstrate a role for c-Src in the earliest stages of ES cell differentiation for the first time. In the present study, we expanded our marker analysis and found that c-Src activity alone also induces ES cell differentiation towards both primitive ectoderm and endoderm, as exemplified by increases in Fgf5, Sox17, Gata4 and Gata6 expression. Moreover, c-Src activity alone drives the expression of EMT markers, consistent with a previous report of Src-mediated EMT during induction of ES cell differentiation through the Calcineurin-NFAT pathway[26].

Here we also describe an analogous 'gatekeeper' mutant of c-Yes that is resistant to A-419259. Unlike c-Src, ES cells expressing this c-Yes mutant did not differentiate in response to inhibitor treatment, and instead retained the same pluripotency gene expression pattern as control ES cells. Interestingly, when inhibitor-resistant mutants of both c-Yes and c-Src were co-expressed in ES cells, c-Yes activity prevented the induction of both endoderm and EMT marker expression by c-Src. These observations suggest the following model of c-Src and c-Yes kinase regulation during ES cell renewal and differentiation[20]. In presence of LIF and serum, both c-Yes and c-Src are active, but the presence of active c-Yes overrides the c-Src signal for differentiation. Upon LIF withdrawal, c-Yes activity is shut off, allowing active c-Src to drive differentiation. More broadly, our work suggests that although ES cells can be maintained in a perpetual state of self-renewal, they are poised to differentiate. Other studies have established that key differentiation genes are transcriptionally initiated in self-renewing ES cells, but are silenced by the renewal-associated transcriptional regulators Oct4, Nanog and Sox2[11]. The ability of c-Yes kinase activity to prevent the transcriptional silencing of these master regulators of self-renewal as shown here may explain its potent suppressive effect on ES cell differentiation.

A long-standing tenet of the Src-family kinase field is that individual family members have similar, if not redundant, biological functions. Our results clearly demonstrate that this is not always the case, and provide an important caveat to the use of broad-spectrum, small molecule inhibitors of all Src kinases to make conclusions about the biological activity of individual family members or the family as a whole. Finally, our results provide a strong rationale for the development of selective compounds to control c-Src vs. c-Yes activity; such compounds may allow more precise pharmacological manipulation of ES cell renewal and differentiation. Indeed, a recent unbiased chemical library screen identified broad-spectrum Src-family kinase inhibitors as potent enhancers of somatic cell reprogramming to an ES cell-like state (iPS cells)[28]. Our work predicts that a Src-selective inhibitor (or a c-Yes agonist) may provide an even greater enhancement in reprogramming efficiency.

METHODS

Cell culture

Rat2 fibroblasts and the mouse ES cell line D3 were obtained from the ATCC and maintained as described previously[20,29]. For inhibitor treatment, ES cells (10^6) were plated on gelatin-coated 60 mm plates for 24 h. A-419259 (Santa Cruz Biotechnology) was added to the culture medium to a final concentration of 1 μM using DMSO as the carrier solvent (0.1% final)[22].

Retroviral transduction

Kinase-dead (Lys295 to Arg), active (Yes-Tyr527 to Phe; Yes-YF), and inhibitor-resistant (Thr338 to Met; Yes-IR) mutants of c-Yes were generated by site-directed mutagenesis (QuickChange XL method; Stratagene). Residues are numbered according to the crystal structure of c-Src (PDB: 2SRC)[30]. The c-Yes cDNAs were subcloned into pMSCV-IRES-Neo and pMSCV-IRES-Puro (Clontech) using the In-Fusion cloning method (Clontech). Analogous retroviral expression vectors for c-Src have been described elsewhere[22]. Production of retroviral stocks and infection of ES cells has been described[22, 31]. Transduced ES cell populations were selected with 250 μg/ml G418, 1.5 μg/ml puromycin or both for the doubly transduced cells. Retroviral transduction of Rat2 cells is described elsewhere[29].

Embryoid body formation

Embryoid bodies were cultured and stained with DAPI as previously described[20]. EB size was estimated from the area of 2D projections of the confocal images[32].

RT-PCR analysis

Total RNA was isolated with the RNeasy Plus Mini Kit (Qiagen) and cDNA was synthesized from 2 μg total RNA with a random decamer as primer according to the manufacturers' protocols (Ambion). For RT-PCR to confirm c-Yes and c-Src transgene expression, one-twentieth of each RT reaction was used in a 50 μl PCR reaction with virus-specific primers (5 μM). Quantitative RT-PCR (qPCR) reactions consisted of 1 μl of a 1:50 dilution of the cDNA reaction, primers (5 μM) and RT2 SYBR green qPCR master mix (Qiagen). Primer sets were obtained from Qiagen (Quantitative Primer Assays), and results were normalized using GAPDH as the reference transcript. Quantitative RT-PCR data were analyzed using the pairwise Fixed Reallocation Randomization Test and the REST 2009 software[33]. Primer efficiency was set to a value of 1 and 5,000 iterations were used to calculate changes in expression and statistical significance. Results are reported as fold change in expression ± S.E.M.

Immunoprecipitation and immunoblotting

Cells were washed with phosphate-buffered saline (PBS) and lysed in RIPA buffer as described[20]. The c-Yes proteins were immunoprecipitated with a c-Yes antibody (BD Biosciences, Cat. # BD610375) and protein G-Sepharose (Invitrogen) and separated by SDS-PAGE. Proteins were transferred to PVDF membranes and probed with antibodies to the c-Yes activation loop (Invitrogen 44660G) and the c-Yes protein (Abcam, AB13954), while blots of the lysates were probed with antibodies to Oct4 (Santa Cruz, SC-5279) and actin (Millipore, MAB1501).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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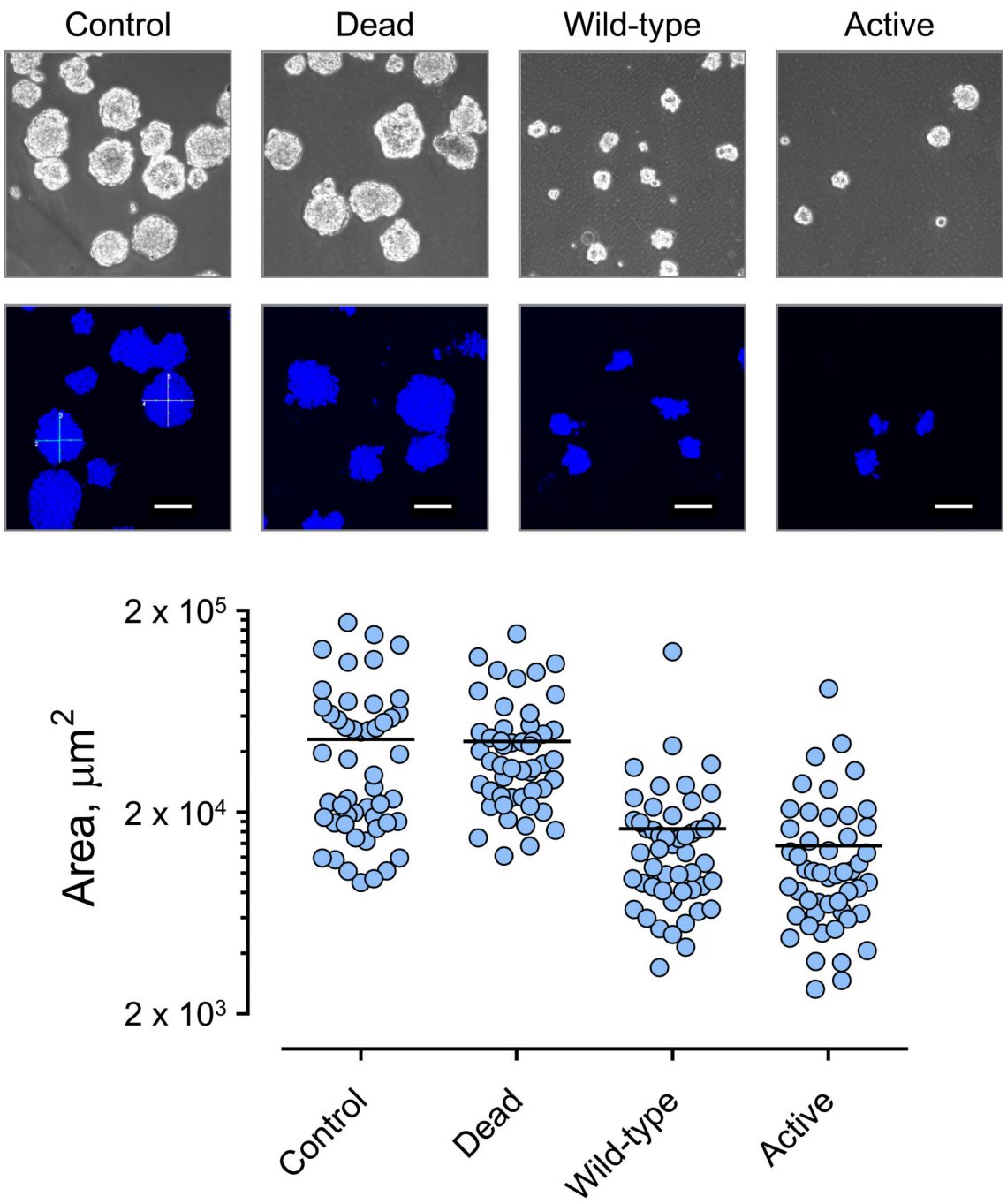

Figure 1. mES cells expressing active c-Yes kinases fail to form EBs

ES cell populations expressing wild-type, active and dead forms of c-Yes as well as the vector control were plated in EB formation assays and imaged 6 days later (top row, bright-field images; bottom row, confocal images of DAPI-stained cultures; scale bar 100 μm). Size estimates for 50 EBs derived from each mES cell population are shown with the median size indicated by the black bar. This experiment was repeated twice with comparable results; a representative example is shown. Unpaired Student's t-tests showed no difference in the average sizes of the EBs formed from the control vs. kinase-dead cell populations or between the wild-type vs. active c-Yes populations. However, both the wild-type and active

c-Yes cell populations formed EBs that were statistically smaller than the control population ($p < 0.0001$).

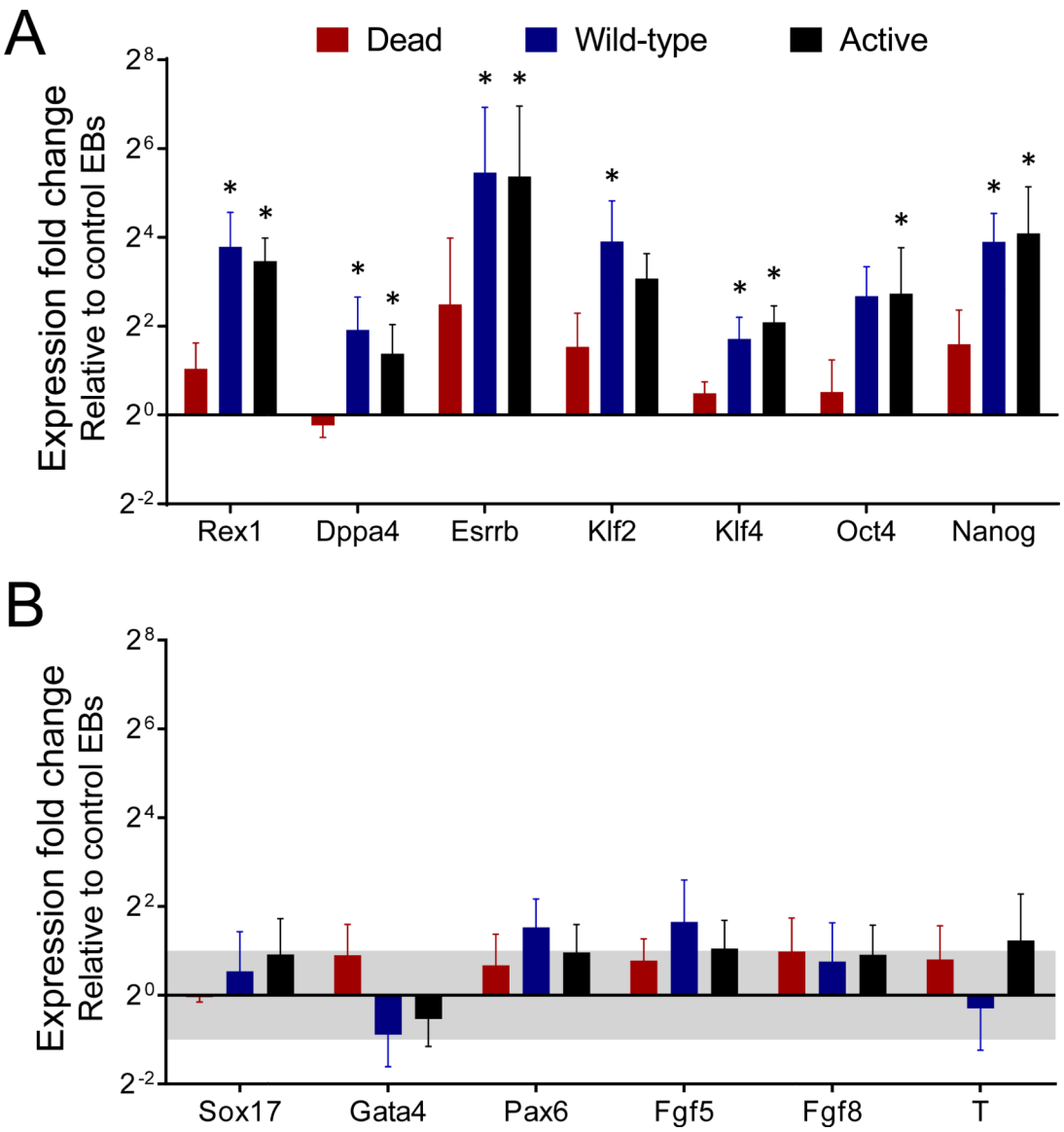

Figure 2. EBs formed from mES cells expressing active c-Yes kinases retain pluripotency marker expression

ES cell populations expressing wild-type, active and dead forms of c-Yes as well as vector control cells were cultured in EB assays for 6 days followed by qPCR analysis with primers specific for pluripotency (A) and differentiation (B) markers. The results are expressed as the average fold-change in marker level in EBs derived from c-Yes-transduced ES cells relative to control EBs ± S.E.M. (n=3, *$p < 0.05$, Pairwise Fixed Reallocation Randomization Test.) The lineages represented by each of the differentiation markers are as follows: Sox17 and GATA4, endoderm; Pax6, ectoderm; Fgf5, Fgf8, ectoderm/epiblast; T, mesoderm. Differentiation marker expression (B) varied by roughly two-fold or less across EBs formed from all four ES cell populations (grey area).

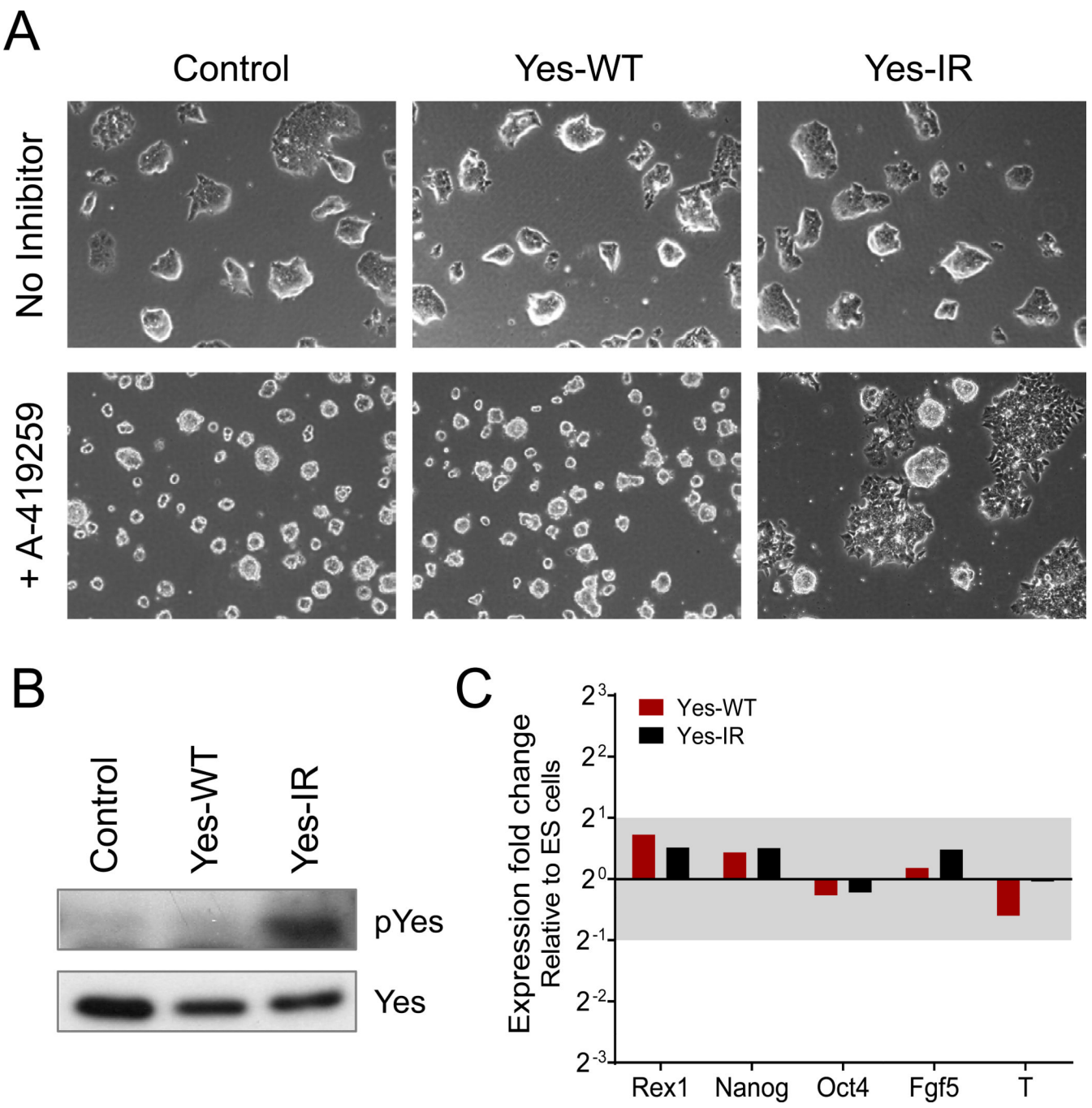

Figure 3. Stable expression of inhibitor-resistant c-Yes in ES cells

A) ES cells were infected with c-Yes wild-type (Yes-WT), Yes-IR or control retroviruses and selected with G418. Cells were cultured with LIF ± A-419259 (1 μM) for 48 hours. Representative bright-field images are shown. Magnification; 100X. B) ES cell populations from part A were treated with A-419259 for 48 hours and c-Yes proteins were immunoprecipitated and blotted with a phosphospecific antibody (pYes) and for c-Yes protein recovery. C) ES cell populations from Part A were cultured with LIF and A-419259 for 4 days, followed by qPCR analysis of the markers shown. Results are expressed as fold change between the ES cell populations expressing c-Yes relative to control cells. Markers

expression varied by less than two-fold in each case (grey area). This experiment was repeated twice with comparable results; a representative example is shown.

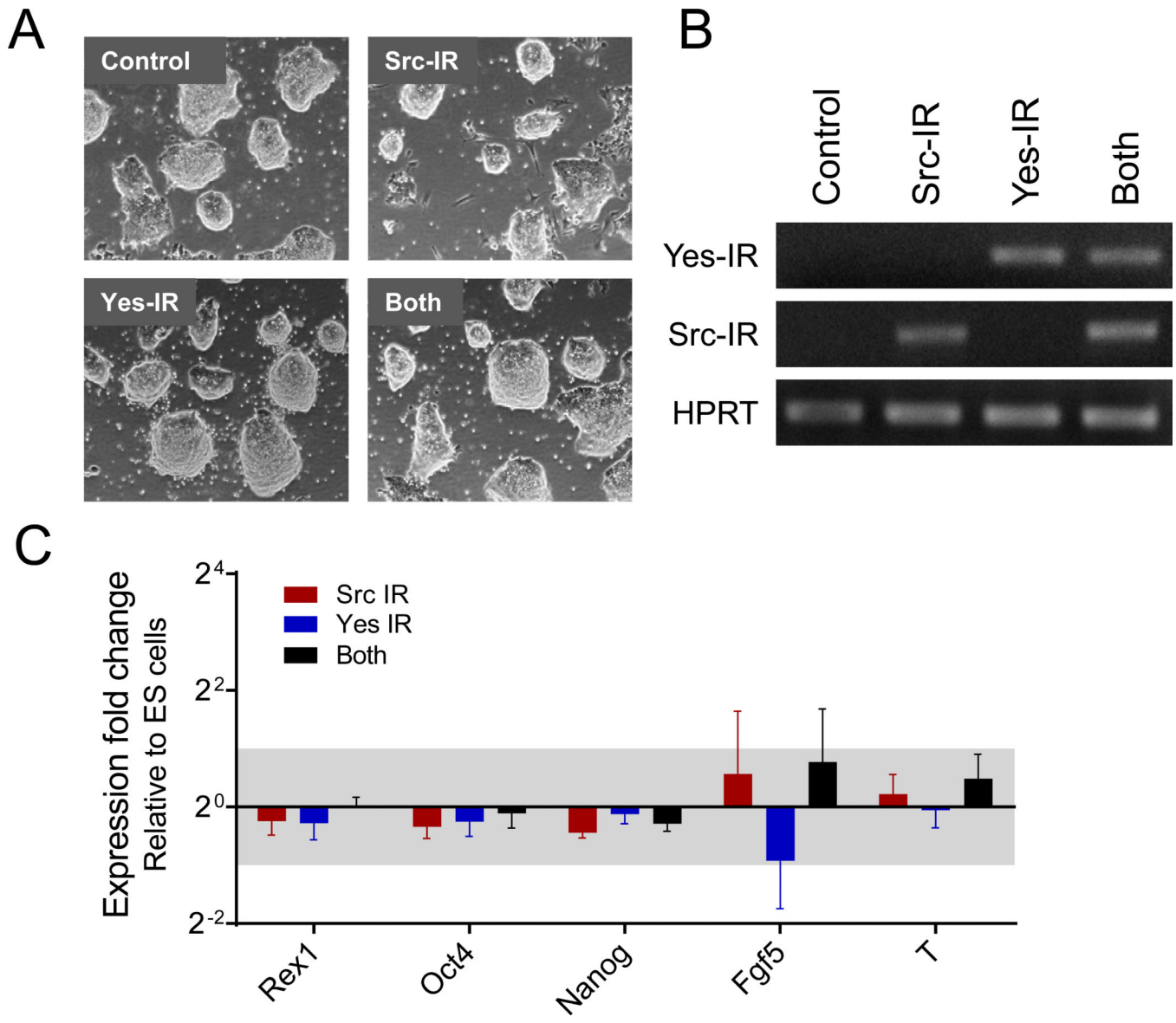

Figure 4. Co-expression of inhibitor-resistant mutants of c-Yes and c-Src in ES cells does not affect self-renewal or differentiation marker expression in the absence of A-419259 treatment

A) ES cell populations expressing the A-419259-resistant mutants of c-Src (Src-IR), c-Yes (Yes-IR), or both were cultured with LIF for 48 hours. Cells transduced with the corresponding empty retroviral vectors served as the negative control. Representative bright-field images are shown. Magnification; 100X. B) Vector-derived Yes-IR and Src-IR expression was confirmed by RT-PCR with vector-specific primers. A scanned image of the resulting agarose gel shows the expected 819 base-pair product for Src-IR and the 604 base-pair product for Yes-IR. An HPRT fragment was amplified as a positive control. C) Expression of the pluripotency markers Rex1, Nanog and Oct4 and the differentiation markers Fgf5 and T was determined in each ES population by qPCR. The analysis was performed in triplicate, and results are expressed as the fold change relative to control ES cells ± S.E.M. Marker expression varied by less than two-fold in each case (grey area).

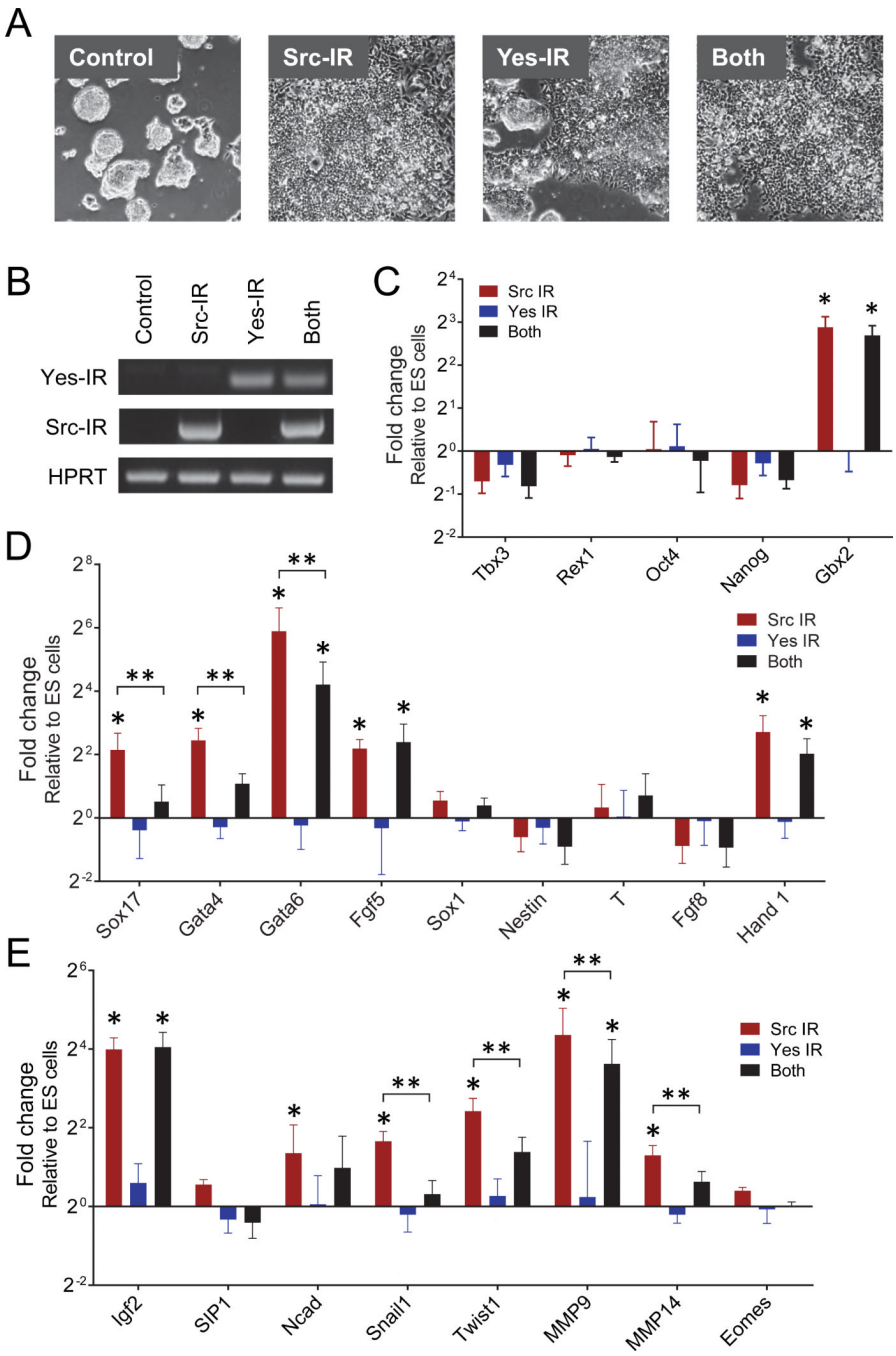

Figure 5. Differentiation of mES cells driven by c-Src is antagonized by c-Yes

A) ES cell populations expressing the inhibitor-resistant mutants of c-Src (Src-IR), c-Yes (Yes-IR) or both were cultured in the presence of LIF and A-419259 (1 μM). Cells transduced with the corresponding empty retroviral vectors served as the negative control. Representative bright-field images were recorded 4 days later. Magnification; 100X. B) Vector-derived Yes-IR and Src-IR expression after inhibitor treatment was confirmed by RT-PCR with vector-specific primers. A scanned image of the resulting agarose gel shows the expected 819 base-pair product for Src-IR and the 604 base-pair product for Yes-IR. An HPRT fragment was amplified as a positive control. qPCR was performed with primers specific for markers of pluripotency (C), differentiation (D) and EMT (E) on each of the cell

populations shown in part A. The analysis was performed on four replicates from two independently derived sets of ES cell populations, and results are expressed as the fold change relative to the control ES cell population ± S.E.M. (*$p < 0.05$ compared with control ES cells; **$p < 0.05$ compared with Src-IR cells; Pairwise Fixed Reallocation Randomization Test.)