Antiproliferative small-molecule inhibitors of transcription factor LSF reveal oncogene addiction to LSF in hepatocellular carcinoma

Trevor J. Grant[a,1], Joshua A. Bishop[b,1,2], Lisa M. Christadore[b], Girish Barot[a], Hang Gyeong Chin[c,d], Sarah Woodson[a], John Kavouris[b], Ayesha Siddiq[e], Rachel Gredler[e], Xue-Ning Shen[e], Jennifer Sherman[a,f], Tracy Meehan[a], Kevin Fitzgerald[f], Sriharsa Pradhan[d], Laura A. Briggs[g], William H. Andrews[g], Devanand Sarkar[e], Scott E. Schaus[b], and Ulla Hansen[a,c,3]

Departments of [a]Biology and [b]Chemistry, Center for Chemical Methodology and Library Development at Boston University, and [c]Program in Molecular Biology, Cell Biology, and Biochemistry, Boston University, Boston MA 02215; [d]New England BioLabs, Inc., Ipswich, MA 01938; [e]Department of Human and Molecular Genetics, Virginia Commonwealth University School of Medicine, Richmond, VA 23298; [f]Alnylam Pharmaceuticals, Inc., Cambridge, MA 02142; and [g]Sierra Sciences, LLC, Reno, NV 89502

Edited by Jonathan R. Hart, The Scripps Research Institute, La Jolla, CA, and accepted by the Editorial Board January 31, 2012 (received for review January 3, 2012)

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide. Despite the prevalence of HCC, there is no effective, systemic treatment. The transcription factor LSF is a promising protein target for chemotherapy; it is highly expressed in HCC patient samples and cell lines, and promotes oncogenesis in rodent xenograft models of HCC. Here, we identify small molecules that effectively inhibit LSF cellular activity. The lead compound, factor quinolinone inhibitor 1 (FQI1), inhibits LSF DNA-binding activity both in vitro, as determined by electrophoretic mobility shift assays, and in cells, as determined by ChIP. Consistent with such inhibition, FQI1 eliminates transcriptional stimulation of LSF-dependent reporter constructs. FQI1 also exhibits antiproliferative activity in multiple cell lines. In LSF-overexpressing cells, including HCC cells, cell death is rapidly induced; however, primary or immortalized hepatocytes are unaffected by treatment with FQI1. The highly concordant structure–activity relationship of a panel of 23 quinolinones strongly suggests that the growth inhibitory activity is due to a single biological target or family. Coupled with the striking agreement between the concentrations required for antiproliferative activity (GI_{50}s) and for inhibition of LSF transactivation (IC_{50}s), we conclude that LSF is the specific biological target of FQIs. Based on these in vitro results, we tested the efficacy of FQI1 in inhibiting HCC tumor growth in a mouse xenograft model. As a single agent, tumor growth was dramatically inhibited with no observable general tissue cytotoxicity. These findings support the further development of LSF inhibitors for cancer chemotherapy.

transcription factor inhibitor | xenograft tumor model | CP2 | *TFCP2*

Hepatocellular carcinoma (HCC) is characterized by late-stage diagnosis and a poor prognosis for treatment, which usually consists of surgical resection of the tumor and chemotherapy (1–3). Currently, the only approved treatment for primary malignancies is sorafenib, a receptor tyrosine kinase and Raf inhibitor originally developed for primary kidney cancer that is also marginally effective against HCC, increasing survival by 2–3 mo as a single treatment. Molecularly targeted therapies are being pursued for a number of signaling proteins of significance in HCC (4–6). Small-molecule inhibitors of oncogenic signaling proteins (e.g., kinases) can provide tremendous therapeutic benefit, due to the phenomenon of oncogene addiction (7, 8). Oncogene addiction, although not well understood at the molecular level, has been characterized as cancer cells being uniquely dependent on an oncogene for continued cell growth and survival. Therefore, upon treatment targeted at an oncogene, cancer cells die, whereas normal cells are spared or only minimally affected.

We demonstrated that the transcription factor LSF can function as an oncogene for HCC (9), also recently confirmed by others (10). LSF, a member of a small family of transcription factors conserved throughout the animal kingdom (11), is ubiquitously expressed in mammalian tissues and cell lines (12). LSF activity is tightly controlled as cells progress from quiescence into DNA replication (G0 to S) (13, 14), and is required for efficient progression of cells through the G1/S transition (15, 16). Regulation of LSF activity normally occurs via posttranslational modifications, with LSF protein levels generally being low and constant. However, LSF protein levels are highly up-regulated in tumor cells, particularly in HCC cell lines and patient samples (9, 17). These elevated LSF levels promote oncogenesis in the HCC cells.

Until recently, transcription factors were generally considered to be undruggable, in particular with respect to obtaining small molecules that specifically inhibit DNA-binding activity. However, such small molecule inhibitors are now being identified for an increasing number of transcription factors (18), with some targeting oligomerization domains, and others directly targeting specific DNA-interaction surfaces. The DNA-binding domain of the LSF transcription factor family is uncommon, with no apparent amino acid similarity to any other proteins (19). Structurally, it is predicted to be similar only to the DNA-binding domain of the p53 family of transcription factors (20). LSF is predominantly dimeric in solution, but tetrameric upon interacting specifically with DNA (21–23), potentially permitting inhibition by either mode.

Here, we identify a family of small molecules that specifically targets the DNA-binding and corresponding transcriptional activities of LSF and that inhibits proliferation of a number of cancer cell lines. The lead LSF inhibitor, factor quinolinone inhibitor 1 (FQI1), rapidly induces apoptosis in an aggressive HCC cell line in vitro and significantly inhibits tumor growth in

Author contributions: T.J.G., J.A.B., L.M.C., H.G.C., J.K., D.S., S.E.S., and U.H. designed research; T.J.G., J.A.B., L.M.C., G.B., H.G.C., S.W., J.K., A.S., R.G., X.-N.S., J.S., and T.M. performed research; K.F., S.P., L.A.B., and W.H.A. contributed new reagents/analytic tools; T.J.G., J.A.B., L.M.C., D.S., S.E.S., and U.H. analyzed data; and S.E.S. and U.H. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission. J.R.H. is a guest editor invited by the Editorial Board.

Data deposition: The description of the synthesis, characterization, and structures of all compounds reported in this paper have been detailed in the *SI Text*.

[1]T.J.G. and J.A.B. contributed equally to this work.

[2]Present address: Center for Human Genetic Research, Massachusetts General Hospital, Boston, MA 02114.

[3]To whom correspondence should be addressed. E-mail: uhansen@bu.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1121601109/-/DCSupplemental.

a mouse xenograft model, with no observable toxicity to normal tissues. These data strongly suggest oncogene addiction of HCC cells to LSF and support the feasibility of directly targeting this transcription factor for chemotherapeutic intervention.

Results

Family of Quinolinones Induces Potent and Specific Inhibition of DNA-Binding and Transcription Activities of the Transcription Factor LSF. Compound hits from a previous screen that aimed to identify small-molecule inhibitors of LSF binding to DNA included several 4-aryl-3,4-dihydroquinolin-2(1*H*)-ones. In the screen, 110,000 commercially available compounds had been evaluated by a fluorescence polarization assay for their ability to diminish LSF DNA binding in vitro. Furthermore, to remove nonspecific inhibitors of protein–DNA interactions, a secondary EMSA had established the compounds that inhibited only LSF DNA-binding activity, and not that of three other transcription factors (Sp1, Oct1, and E2F3). These widely expressed proteins represent a range of DNA-binding domains—zinc finger, POU homeodomain, and winged helix, respectively. Thus, 4-aryl-3,4-dihydroquinolin-2(1*H*)-ones generally had emerged as inhibitors of LSF that were both potent and specific, at least in purified in vitro DNA-binding systems. Before synthesizing additional compounds, we had also determined that commercially available quinolinones that inhibited LSF DNA-binding activity in vitro also inhibited LSF transcriptional activity in a cellular context, using an LSF-dependent luciferase reporter assay.

Thus, a collection of compounds based on the dihydroquinolin-2(1*H*)-one structure was prepared (24) (*SI Appendix*). The ability of these small molecules to inhibit LSF activity was initially evaluated at 0.5, 1.0, and 5.0 μM in NIH 3T3 cells, using a LSF-dependent luciferase reporter assay. In the first panel of compounds, FQI1 (Fig. 1*A*) was identified as one of the most active compounds in stimulating LSF-driven firefly luciferase expression from an LSF-dependent reporter construct (*SI Appendix*, Fig. S1). With a more extensive titration curve, the concentration at which LSF transactivation was inhibited 50% (IC_{50}) was determined to be 2.1 μM (Fig. 2*A* and Table 1). That transcriptional inhibition by FQI1 was specific to LSF was confirmed in several ways. First, firefly luciferase activity derived from the LSF-dependent reporter construct (Fig. 2*A*, *Upper*) was normalized in all cases to the activity of an internal control, expression from a renilla luciferase gene driven by the Herpes simplex viral thymidine kinase (HSV- tk) promoter. Expression from this internal control was invariant with FQI1 treatment, demonstrating that the transcription factors that bind and stimulate transcription from this promoter were unaffected by FQI1. Second, in a parallel experiment, FQI1 did not affect the degree of expression from a USF-dependent reporter construct, activated by USF expression (Fig. 2*A*). Finally, in a more stringent test of FQI1 specificity, the activity of a transcription factor whose DNA-binding domain is similar to that of LSF was assayed. Although there is no known protein structure for members of the LSF transcription factor family, and no apparent protein sequence homology outside of this small family to the LSF DNA-binding region (19), one report used a variety of protein-folding algorithms to predict that the DNA-binding region is similar in structure to that of the p53 transcription factor family (20). Our structure–function analyses of LSF DNA binding are consistent with this prediction (13). Thus, we tested whether p53-mediated transcription would be

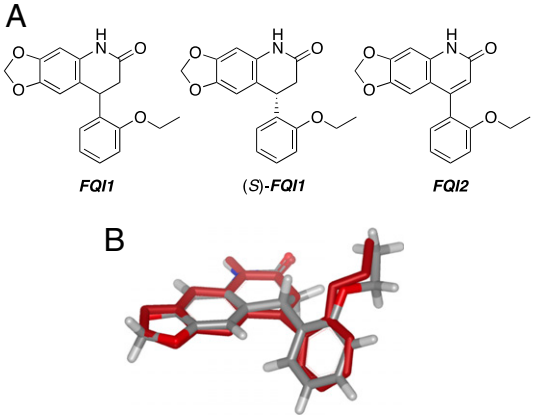

Fig. 1. Structures of LSF inhibitors. (*A*) *FQI1* was initially identified as the racemate. The more-active *S* enantiomer, (*S*)-*FQI1*, and the achiral quinolinone inhibitor *FQI2* possess similar chemical properties and biological activities. (*B*) A computationally generated overlay of (*S*)-*FQI1* (cpk) and FQI2 (red) using the OpenEye Scientific Software shape-similarity comparison program ROCS. Although achiral, *FQI2* is capable of adopting similar conformations as (*S*)-*FQI1*.

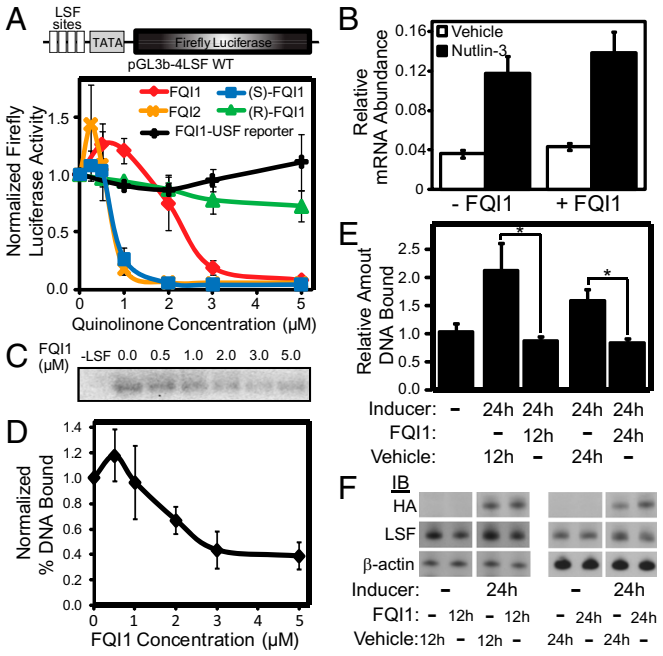

Fig. 2. FQI compounds inhibit LSF transcriptional activation and DNA binding. (*A*) LSF-dependent firefly luciferase reporter activities in NIH 3T3 cells from transfection using the indicated reporter construct (*Upper*), an LSF expression construct, and an internal control. For each concentration of FQI, firefly luciferase activities were normalized to activity in the absence of FQIs. Each of the four FQIs was tested independently. The USF-dependent reporter assays were performed with FQI1 only. Averages with SD derive from 3–4 independent experiments. (*B*) Induction of p21 mRNA levels by nutlin-3 in U2OS cells was assayed in the presence and absence of 5 μM FQI1. Shown are averages of three independent experiments, with SD. (*C*) Representative EMSA of LSF/DNA complexes from in vitro-translated LSF incubated with FQI1. –LSF, translation extract with no programmed LSF. (*D*) Fraction of radiolabeled DNA bound to LSF, normalized to bound levels in absence of FQI1, were averaged (with SEM) from three EMSAs. (*E*) ChIP with an anti-HA antibody of tagged LSF binding to endogenous *POLA1* promoter. Cells were treated with the inducer (RSL1) for 24 h to cause expression of Myc-LSF-HA. In induced cells, FQI1 or vehicle (DMSO) was added for the entire or final half of the induction period. For the uninduced samples, cells treated with vehicle or FQI1 were averaged together. Data are averages of three independent experiments (with SEM). *$P < 0.05$. (*F*) Immunoblotting for total LSF, myc-LSF-HA, and β-actin in cells treated as indicated. Quantitation showed a subtle decrease in total LSF levels upon treatment with FQI1 for 12 h, but no statistically significant decrease upon 24-h treatment. No difference in HA-tagged LSF levels was detected.

Table 1. Inhibitory concentrations for cell proliferation and LSF-dependent reporter studies

Compound	IC_{50} NIH 3T3 LSF-dependent reporter assay, μM*	GI_{50}, μM*		
		NIH 3T3	HeLa	A549
FQI1	2.1 ± 0.21	3.8 ± 0.3	0.79 ± 0.04	6.3 ± 1.1
(*R*)-FQI1	>10	52 ± 3	7.9 ± 1.0	25 ± 2
(*S*)-FQI1	0.93†	0.82 ± 0.2	0.30 ± 0.03	1.2 ± 0.3
FQI2	0.71 ± 0.19	0.71 ± 0.08	0.40 ± 0.04	1.6 ± 0.7

*Error bars represent 95% confidence intervals.
†The curve is too sharp to fit the model, thus confidence intervals not determinable.

influenced by FQI1 treatment. Treatment of cells with nutlin-3, a molecule that increases levels of p53 by inhibiting MDM2 (25), resulted in p53-mediated transcriptional induction of the p21 gene (Fig. 2*B*). Addition of FQI1, at levels that abolish LSF activity, had no effect on activation of p21 expression using this paradigm. These results, taken together, support the high degree of specificity of FQI1 toward inhibiting LSF.

Using FQI1's structure as a starting framework, we sought to determine whether inhibition of LSF was unique to the racemate or if restricting the orientation of the aryl substituent altered the molecule's potency against LSF. First, FQI1 was separated by chiral chromatography to the corresponding *R* and *S* enantiomers (Fig. 1*A*). The luciferase reporter assay revealed significant enantiomeric specificity, in that the (*R*)-FQI1 was much less active in inhibiting LSF transcriptional activity than the racemate, whereas (*S*)-FQI1 was approximately twofold more active than the racemate (Fig. 2*A* and Table 1). Next, we postulated that the achiral quinolin-2(1*H*)-one, FQI2, could adopt a similar conformation as the *S*-enantiomeric counterpart (26), thereby acting as a strong inhibitor of LSF. Unsaturation of the quinolinone ring would still allow rotation about the C8 carbon–carbon bond displaying the 2-ethoxyphenyl substituent, giving rise to similar conformations (Fig. 1*B*). Indeed the activity of FQI2 against LSF in the luciferase assay proved to be as effective as the *S* enantiomer; both were functional at submicromolar concentrations (Fig. 2*A*). This result indicated that the inhibitor's interaction with its molecular target is distinctly dependent on the planar arrangement of the bulky aryl substituent.

An unanticipated result from the LSF-dependent reporter assays was the unconventional activity trend of the potent FQIs. The FQIs reproducibly stimulated LSF transcriptional activity at low concentrations, and then sharply reduced activity as FQI concentrations increased. This behavior suggested allosteric control of LSF by the FQIs. Because the DNA-binding moiety of LSF is tetrameric, we hypothesize that at subsaturating levels, binding of FQIs to LSF monomers results in conformational changes that enhance LSF activity. Only at saturating levels of FQIs is LSF activity ablated. Similar modulation of LSF DNA binding by FQI1 was observed in vitro using EMSAs (Fig. 2 *C* and *D*), with enhancement at low concentrations and inhibition at higher concentrations. Although these results mirrored the concentration-dependent effects of FQI1 on LSF-mediated transactivation, the extent of inhibition of DNA binding in vitro was not as pronounced as inhibition of LSF transactivation in the cell-based luciferase reporter assay. LSF activity in both assays requires tetramerization.

Nonetheless, the simplest mechanism to explain how LSF transcriptional activity is inhibited by FQI1 in vivo would be through the inhibition of LSF binding to DNA in the cellular context. To test this hypothesis, we performed ChIP experiments. In particular, we monitored the binding of an inducible, tagged LSF to an endogenous cellular target promoter in the presence and absence of FQI1. A stable cell line expressing an artificial heterodimeric nuclear receptor (27) was generated in which expression of HA-LSF-myc was induced by the nonsteroidal diphenylhydrazine compound RSL1. In this cellular system, there was no background expression of the inducible protein in the absence of RSL1 (Fig. 2*F*, HA immunoblot), and the level of induced, tagged LSF was comparable to the level of endogenous LSF (Fig. 2*F*, LSF immunoblot), making it ideal for such experiments. As expected, induction of binding of HA-LSF-myc to the promoter of *POLA1*, encoding the catalytic subunit of DNA polymerase α (28, 29), was observed upon treatment with RSL1. FQI1 treatment for the final 12 h or for the entire 24-h induction period efficiently blocked the binding of LSF to the promoter (Fig. 2*E*). Because levels of HA-tagged LSF remained comparable with treatment of FQI1 vs. vehicle (Fig. 2*F*), the differences in binding to the endogenous promoter must be due to differences in DNA-binding affinity.

LSF Small-Molecule Inhibitors also Inhibit Cell Proliferation. Given previous demonstrations that LSF activity is essential for G1/S progression (15, 16), we determined the extent to which the compounds in the FQI library inhibited cell proliferation. The lead compound, FQI1, inhibited growth of multiple cell lines. In particular, the number of viable NIH 3T3, HeLa S3, and A549 cells was reduced by 50% at concentrations of 3.8, 0.79, and 6.3 μM (GI_{50}s), respectively (Table 1). Coinciding with the observations in the luciferase reporter assay, the *R* enantiomer was >10× less effective, whereas the *S* enantiomer was at least twice as effective, as the FQI1 racemate in each cell line. Similar to the effects on LSF transactivation, the achiral quinolinone inhibitor, FQI2, was as active as (*S*)-FQI1 at inhibiting cell growth. Strikingly, the half-maximal concentrations for growth inhibition of NIH 3T3 cells of the most potent FQI compounds approximated, considering experimental error, the half-maximal concentrations for inhibition of LSF transcriptional activity measured by the luciferase reporter assay in these same cells [Table 1, *SI Appendix, Cellular Growth Inhibition Assays (A549, HeLa, NIH-3T3 Cells)*, and *SI Appendix*, Table S1].

To test whether the antiproliferative effects resulted from specific or broad target specificity, structure–activity relationship (SAR) analysis was performed by comparing GI_{50} values from all of the FQI analogs containing combinations of peripheral structural variations. The quantitative, directional changes in GI_{50} values caused by each specific modification were consistent across cell growth and LSF transcriptional activity inhibition assays in all cell lines, regardless of peripheral modifications to the core structure (*SI Appendix*, Table S2). The high degree of specificity inferred from this remarkably concordant SAR strongly suggests that the FQI antiproliferative phenotypes result from targeting a single, or highly related, molecular target(s). Combined with the striking agreement between the FQI concentrations required to inhibit both cell proliferation and LSF transactivation, these results indicated that the biological target of FQIs is almost certainly the LSF family of transcription factors.

Potent LSF Small-Molecule Inhibitors Cause Rapid Cell Death in Liver Cancer Cell Lines. Given the oncogenic properties of LSF, we also examined the effect of FQI1 on proliferation of cancer cell lines in which LSF is significantly overexpressed (e.g., HCC cells).

In particular, QGY-7703 is an aggressive HCC cell line that expresses LSF at highly elevated levels. Expression of a dominant-negative mutant of LSF in QGY-7703 cells is sufficient to reduce growth of this cell line in vitro and to reduce tumor growth in xenograft models (9). In cell viability assays [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)], both FQI1 and FQI2 decreased overall cellular proliferation of QGY-7703 cells (Fig. 3*A*), as anticipated from effects in other cell lines. Additionally, cell viability actually dropped, most apparently in the QGY-7703 cells between 24 and 48 h of incubation with FQI1 (Fig. 3*A*). Similar effects were observed in a hepatoblastoma cell line, Hep3B, in which LSF is also overexpressed (9), although requiring a longer time course of treatment (between 48 and 72 h; Fig. 3*B*). These results suggested the possibility that the QGY-7703 cells may be "addicted" to elevated activity of LSF, therefore leading to cell death upon direct targeting of LSF by FQI1. This prediction was verified by TUNEL assays, in which massive apoptosis was observed (Fig. 3*C*) in both QGY-7703 cells (averaging 90% at 48 h of FQI1 treatment) and Hep3B cells (averaging 65% at 96 h of FQI1 treatment). We have previously shown that inhibition of LSF by high expression of dominant-negative LSF in murine fibroblasts or human prostate cancer cells induced apoptosis in S-phase, due substantially to blocking expression of thymidylate synthase (15). However, in the QGY-7703 cells, incubation with thymidine to overcome cellular dependence on thymidylate synthase did not affect the decreased viability upon FQI1 treatment (*SI Appendix*, Fig. S2), indicating that blockage of additional LSF target genes or regulated pathways contributed to cell death.

Finally, we tested whether FQI1 affected normal hepatocytes. Strikingly, no growth consequences or toxicity were observed in either immortalized human hepatocytes (Hc3716-hTERT; Fig. 3*D*) or primary nondividing mouse hepatocytes (Fig. 3*E*). These results clearly demonstrate the distinct FQI1 growth consequences to oncogenic liver cells. This finding is also consistent with the phenomenon of oncogene addiction.

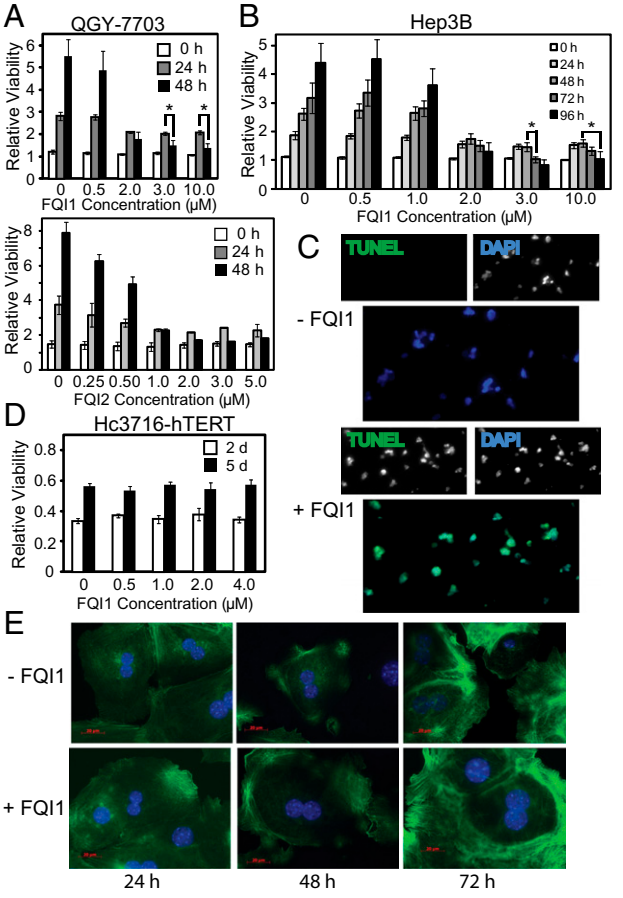

Fig. 3. In vitro growth inhibition and induction of apoptosis in HCC cells, but not hepatocytes, by FQI compounds. (*A*) Relative cell viability of QGY-7703 HCC cells, treated with FQI1 (*Upper*) or FQI2 (*Lower*) for the indicated times, was assayed by MTT. Averages with SEM of three experiments are shown. $*P < 0.05$ by one-tailed *t* test. (*B*) Relative cell viability of Hep3B cells, treated with FQI1 for the indicated times, as assayed by MTT. Averages with SEM of 3–6 independent experiments are shown. $*P < 0.05$ by one-tailed *t* test. (*C*) QGY-7703 cells treated with vehicle or FQI1 were imaged separately for fluorescence resulting from TUNEL positivity or DNA (DAPI). Larger panels below split panels show merged images, with TUNEL positivity as green and DAPI as blue. (*D*) Viability of immortalized human hepatocytes (Hc3716-hTERT), treated with FQI1, was assayed by MTT. Error bars represent SEM. (*E*) Newly plated primary mouse hepatocytes, treated with or without FQI1 for the indicated times, were stained for DNA and F-actin. Note that binucleate cells are common for adult hepatocytes.

FQI1 Inhibits HCC Tumor Growth in Mouse Xenografts, with No General Toxicity. Induction of apoptosis by the specific LSF inhibitors in the QGY-7703 cells in vitro suggested utility for such compounds in treatment of tumors in vivo. In an s.c. xenograft model, mice with small QGY-7703–derived tumors were given multiple, spaced i.p. injections of FQI1 over a 2-wk period, followed by an additional 2 wk without treatments. There was a remarkable decrease in tumor growth in the mice injected with FQI1, as measured by endpoint tumor volumes (Fig. 4*A*; ~ninefold) and tumor weights (*SI Appendix*, Fig. S3*A*), and reflected also by decreased vascularity (*SI Appendix*, Fig. S3*B*). When the endpoint

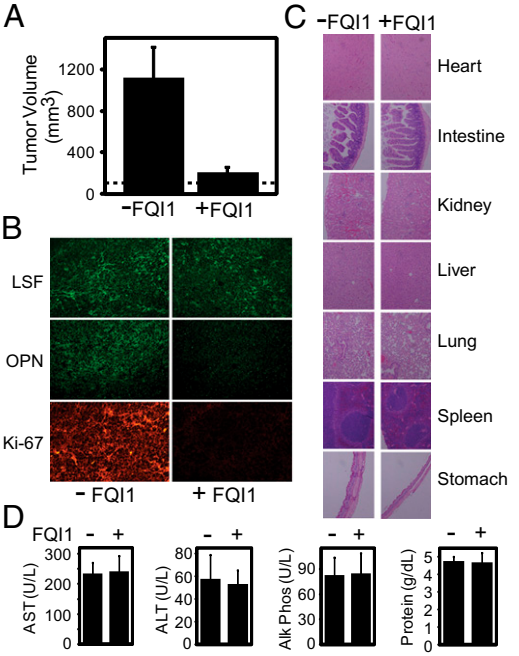

Fig. 4. Inhibition of tumor growth by FQI1 in mouse HCC xenografts. (*A*) Mice containing s.c. QGY-7703 xenografts were treated with or without FQI1 (10/group), and tumor volumes measured 2 wk following treatment. Dashed line: average tumor volume before treatment. (*B*) Fixed tumor sections from mouse xenografts after treatments were assayed for LSF, osteopontin (OPN), and *Ki*-67. (*C*) Representative images (H&E staining) are shown of sections from organs of mice with the indicated treatments. (*D*) Blood from mice with the indicated treatments was analyzed for total protein and activities of aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (Alk Phos). Error bars represent SEM.

tumors were analyzed by immunofluorescence, tumors from FQI1-treated mice expressed LSF at similar levels to those of control mice (Fig. 4*B*). However, expression of osteopontin, whose gene is activated directly by LSF (9), was abolished by FQI1 treatment, as was expression of *Ki*-67, indicative of the replicative capacity of the tumors. These results are consistent with the in vivo inactivation by FQI1 treatment of LSF activities as both a transcription factor and a proliferation driver.

In contrast to the dramatic effects on tumor growth, no general toxicity was evident in the inhibitor-treated animals, as assayed by lack of changes in body weight, feeding, grooming, posture, and general behavior. In addition, H&E staining of multiple tissues showed no discernable toxicity of FQI1, including in the stomach and intestine (Fig. 4*C*). Finally, levels of liver enzymes and protein in the blood were also unaffected, indicating normal liver function (Fig. 4*D*). The tumor-specific effect of this small-molecule inhibitor of the oncogene LSF in mice fits the classic definition of oncogene addiction.

Discussion

LSF is a ubiquitously expressed transcription factor in mammalian cells, whose substantially enhanced expression in hepatocellular carcinoma can promote oncogenesis. We present here a class of small-molecule inhibitors of LSF, named FQIs, as promising candidates for chemotherapy. FQIs target LSF, repressing its DNA-binding activity both in vitro and in vivo, and inhibiting its ability to activate transcription in cells. FQIs do not generally affect RNA polymerase II-mediated transcription or specifically inhibit DNA binding or transcription activities of several other transcription factors that have been tested. In particular, we demonstrated that FQI1 did not affect the activity of p53, representing the only transcription factor family proposed to contain a structurally similar DNA-binding domain to that of LSF.

FQIs also exhibited antiproliferative properties when assayed on a variety of cell lines. Our SAR analysis, combined with the coincidence of concentrations at which both cell growth and LSF transactivation activities were inhibited, strongly suggested that the growth inhibitory activity of FQIs was in fact a consequence of targeting LSF activity. Coincident with our studies, FQI1 was evaluated as a part of the National Institutes of Health Molecular Libraries Initiative as a member of the Small Molecule Repository screened in the Probe Production Network. A search in PubChem (CID 656346) indicated that as of June 27, 2011, FQI1 had been assayed in 575 assays and was determined to be active in 20 of those assays. Interestingly, 3 of these 20 assays were antiproliferative assays performed at the Sanford–Burnham Center for Chemical Genomics [assay IDs (AIDs) AIDs 430, 431, and 620]. Although no inhibitory concentrations were reported, and FQI1 did not lead to a probe series, the AID screens served to independently validate the potential antiproliferative activity of this compound. These and our proliferation studies provide indications that chemical inhibition of LSF by FQIs leads to cancer cell growth inhibition.

Most significantly, FQI1 treatment of HCC cells in culture and of HCC s.c. xenografts in a mouse tumor model point toward these tumor cells being addicted to LSF activity. Whereas the aggressive tumor cells in culture underwent apoptosis, both primary and immortalized hepatocytes in culture were unaffected by treatment. More importantly, tumor growth in mouse xenograft assays was dramatically reduced, but no toxicity was observed in any other tissues or in liver function (as monitored by liver enzymes in blood). Such an ability of an oncogene inhibitor to kill tumor cells without affecting normal cells is the hallmark characteristic of oncogene addiction.

These findings suggest the exciting therapeutic potential for FQIs or other small-molecule inhibitors of LSF in targeting oncogene addiction to LSF in hepatocellular carcinoma. We previously established that LSF was both sufficient and necessary for aggressive HCC tumor growth in mouse xenograft assays, and also that expression levels of LSF in human patient HCC samples correlated with increased stage and decreased differentiation state of disease (9). Taken together with the currently limited treatment options for HCC, the FQI compounds described here are promising candidates for pharmaceutical development for HCC treatment; they may also be effective agents in combating other cancers in which LSF serves as an oncogene.

Materials and Methods

High-Throughput Screen of Compounds That Inhibit LSF DNA Binding. An automated liquid handling (Biomek FX Workstation; Beckman Coulter), 384-well plate sealing and piercing (ABgene), and multimodal plate reading (Envision Plate Reader; PerkinElmer Wallac) integrated high-throughput screen system was used. Purified LSF was incubated in the presence of vehicle or 20-μM compound plates for 10 min at room temperature. BODIPY TMR-X–labeled double-stranded LSF binding site (CGCGGCGCGAGTTTCAGGC and its complement) or control DNA was added to 1 nM, followed by incubation for 45 min at room temperature, in 24 mM Hepes (pH 7.9), 41 mM KCl + NaCl, 2.3 mM $MgCl_2$, 0.0625% Tween 20, 3% (vol/vol) glycerol, 0.0005% octylphenoxypolyethoxyethanol, 0.7 mM EDTA, 0.8 mM DTT, 0.7 mM Tris, 4% DMSO, and 0.4 U/mL polydeoxy(inosinate-cytidylate)•(inosinate-cytidylate). Fluorescence polarization and total fluorescence intensity were measured (30).

Synthesis and Analyses of FQIs. See *SI Appendix* for details.

Cell Culture. NIH 3T3 Mouse fibroblasts were cultured in DMEM with 10% calf serum. Two human carcinoma cell lines, A549 small cell lung and HeLa cervical (ATCC), were grown in MEM with 10% FBS. Human U2-OS cells were cultured in DMEM with 10% FBS. The human QGY-7703 HCC cell line (gift of Zhaozhong Su, Fudan University, Shanghai, China) was cultured in DMEM with 10% FBS. The telomerase-immortalized normal human hepatocyte Hc3716-hTERT cell line (gift of Kumiko Anno, Hiroshima University, Hiroshima, Japan) was cultured as described (31). Primary mouse hepatocytes (32) were isolated from a 10-wk-old C57 female mouse using a two-step perfusion method. Primary hepatocytes (3×10^5), at 96% viability, were plated onto coverslips coated with 0.1% rat tail collagen in six-well plates. Approximately 4 h later, once cells adhered to the coverslips, they were incubated in media with either 5 μM FQI1 or vehicle.

The stable cell line for inducible expression of myc-LSF-HA is based on the RheoSwitch expression system (NEB) (27). The parental HEK293-A7 RheoSwitch cell line (NEB) expresses an engineered nuclear receptor heterodimer for RheoReceptor-1 and RheoActivator, which is stabilized for transcriptional activation by the nonsteroidal diphenylhydrazine compound, RSL1, a specific ligand for this receptor heterodimer (33). Cells were maintained in DMEM with 10% FBS. Cell clones were isolated with the stably integrated expression plasmid pNEBRX1-Myc-LSF-HA (puroR), by growing in media with 1 μg/mL puromycin.

Cell Proliferation. For cell viability assays over time after treatment with FQIs, QGY-7703 and Hep3B cells were seeded in 96-well plates at 1–2 $\times 10^3$ cells/well, and FQI or vehicle (final DMSO of 0.5%) was added at appropriate concentrations 1 h later. At the indicated time points, cell viability was assayed using a standard MTT assay (Promega). Hc3716-hTERT cells were plated at 5,000 cells/well and treated with FQI1 after 1 d.

For SAR assays, A549, HeLa (both at 3×10^4 cells/mL), or NIH 3T3 (1×10^4 cells/mL) cells were seeded in 96-well plates and treated with compound or vehicle (DMSO) at appropriate concentrations (DMSO at 1%) after 24 h (A549 and HeLa) or 18 h (NIH 3T3). After 72 h incubation with compound or vehicle, confluent cells were assayed for growth inhibition using the [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS) assay (Promega). GI_{50} values (*SI Appendix*) were determined from plots of the percentage of compound-treated cell growth to control cell growth vs. compound concentration (Prism GraphPad).

LSF-Dependent Luciferase Reporter Assays. NIH 3T3 cells were transfected for 5 h with pEF1α-LSF (15, 34), the reporter construct pGL3B-WT4E1b (14), phRL-TK, and pEFGP (14). Vehicle (DMSO) or FQI compound was then added, keeping DMSO at 0.5%. Cell extracts were harvested 36 h posttransfection, and firefly and *renilla* luciferase activities measured via a dual luciferase assay (Promega). Relative luciferase activity represents firefly luciferase activity normalized that of *renilla* luciferase in each extract. For testing USF, cells were transfected with pCX-USF1, the reporter construct pGL3-Atrogin1-0.4kb, the inducer pcDNA3-GSKβ S9A, and pGK–β-gal (all from Geoffrey

CELL BIOLOGY

Cooper, Boston University, Boston). Firefly luciferase activity was normalized to β-gal activity in each extract. IC_{50} values were determined from plots of normalized luciferase activity vs. compound concentration (Prism GraphPad).

Electrophoretic Mobility Shift Assays. In vitro-translated LSF (22) was preincubated with inhibitor or DMSO and then incubated with 5 μg/mL polydeoxy(inosinate-cytidylate)•(inosinate-cytidylate) for 15 min at 4 °C in 40 mM KCl, 15 mM Hepes (pH 7.9), 1 mM EDTA, 0.5 mM DTT, and 5% (vol/vol) glycerol. Radiolabeled double-stranded DNA (TGGCTGGTTATGGCTGGTCAGACTAG and its complement) was added for 30 min incubation at 30 °C, and the samples electrophoresed through a native 6% polyacrylamide gel. Results were visualized using a Typhoon imager and quantified using ImageQuant software.

ChIP Assay. The stable HEK293 cell line containing the inducible myc-LSF-HA construct was treated at 60–70% confluency with 500 nM RSL1 (the inducer) and 5 μM FQI1 or vehicle, as indicated. ChIP was performed on formaldehyde-treated cells using the SimpleChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology). Immunoprecipitated DNA (150–350 bp) was analyzed with primers upstream of the *POLA1* gene (CCAAATCTTTTCCCATCAGCA; AGCCGCCTGGAGACTGC) using the MyiQ Real-Time PCR Detection System (BioRad) and DyNAmo HS SYBR Green (NEB).

Immunoblotting. The myc-LSF-HA–inducible cells were treated with RSL1, FQI1, and/or vehicle as indicated. Whole-cell extracts were isolated using 50 mM Hepes (pH 7.5), 250 mM NaCl, 50 mM NaF, 5 mM EDTA, and 1% Triton X-100 buffer, and then electrophoresed through a 10% tricine-SDS/PAGE. For immunoblotting, anti-LSF (BD Biosciences), and anti-HA and anti–β-actin antibodies (Cell Signaling Technology) were used. For quantitation, fluorescent secondary antibodies were used, and the blots analyzed using the LI-COR Odyssey scanner and Quantity One imaging software.

TUNEL Assays. Cells were plated 24 h before incubation with 10 μM FQI1 or vehicle (DMSO). At the indicated time points, cells were suspended, fixed with 80% ethanol, and plated on gelatin/polylysine-coated slides. TUNEL assays were performed using the FragEL DNA fragmentation kit (Calbiochem), visualizing DNA nicks with fluorescein and total DNA with DAPI.

Mouse Xenograft Assays. Subcutaneous xenografts were established in flanks of athymic nude mice using QGY-7703 cells (5×10^5) (9). When tumors reached ~100 mm^3 (about 1 wk), mice were injected i.p. with FQI1 (1 mg/kg) or vehicle diluted in PBS, a total of 5×, once every 3 d. Two weeks after the final treatment, mice were killed. Tumor samples were immunostained (9, 17) using antibodies against LSF (BD Biosciences), OPN (Santa Cruz Biotechnology), and *K*i-67 (BD Biosciences).

Toxicity. For tissue and blood analyses, male nude mice (4-6 wk old) were injected i.p. 5 d/wk for 2 wk with FQI1 (2 mg/kg) or vehicle. One week following treatments, mice were killed. Formalin-fixed paraffin-embedded sections from internal organs were analyzed by H&E staining. Blood collected at the time of sacrifice was subjected to liver function tests. Primary mouse hepatocytes on coverslips were fixed with 4% paraformaldehyde, stained for DNA and F-actin, and imaged using a Zeiss Image Z.1 microscope.

ACKNOWLEDGMENTS. The authors thank Johann Bergholz and Drs. Kumiko Anno, Geoffrey Cooper, and Zhao-zhong Su for kindly providing reagents; M. Servergnini for assistance with primary hepatocyte isolation; B.-K. Yoo for initial tumor xenograft analyses; and the Molecular Diagnostic Laboratory, Department of Pathology, Virginia Commonwealth University, for performing liver function tests. This work was supported by the Boston University Undergraduate Research Opportunity Program (T.J.G. and S.W.); a Mary Erskine award from National Science Foundation Partnerships for Adaptation, Implementation, and Dissemination Grant HRD-0820175 (to U.H.); National Institutes of Health Grants R01 GM078240 and R01 GM078987 (to S.E.S.) and R01 CA138540 (to D.S.); the James S. McDonnell Foundation (D.S.); New England BioLabs (H.G.C.); and Alnylam Pharmaceuticals (J.S.).

1. El-Serag HB, Rudolph KL (2007) Hepatocellular carcinoma: Epidemiology and molecular carcinogenesis. *Gastroenterology* 132:2557–2576.
2. Farazi PA, DePinho RA (2006) Hepatocellular carcinoma pathogenesis: From genes to environment. *Nat Rev Cancer* 6:674–687.
3. Yau T, Chan P, Epstein R, Poon RT (2008) Evolution of systemic therapy of advanced hepatocellular carcinoma. *World J Gastroenterol* 14:6437–6441.
4. Tanaka S, Arii S (2009) Molecularly targeted therapy for hepatocellular carcinoma. *Cancer Sci* 100:1–8.
5. Llovet JM, Bruix J (2008) Molecular targeted therapies in hepatocellular carcinoma. *Hepatology* 48:1312–1327.
6. Whittaker S, Marais R, Zhu AX (2010) The role of signaling pathways in the development and treatment of hepatocellular carcinoma. *Oncogene* 29:4989–5005.
7. Weinstein IB, Joe A (2008) Oncogene addiction. *Cancer Res* 68:3077–3080, discussion 3080.
8. Sharma SV, Settleman J (2010) Exploiting the balance between life and death: Targeted cancer therapy and "oncogenic shock". *Biochem Pharmacol* 80:666–673.
9. Yoo BK, et al. (2010) Transcription factor Late SV40 Factor (LSF) functions as an oncogene in hepatocellular carcinoma. *Proc Natl Acad Sci USA* 107:8357–8362.
10. Fan R-H, Li J, Wu N, Chen P-S (2011) Late SV40 factor: A key mediator of Notch signaling in human hepatocarcinogenesis. *World J Gastroenterol* 17:3420–3430.
11. Traylor-Knowles N, et al. (2010) The evolutionary diversification of LSF and Grainyhead transcription factors preceded the radiation of basal animal lineages. *BMC Evol Biol* 10:101.
12. Swendeman SL, et al. (1994) Characterization of the genomic structure, chromosomal location, promoter, and development expression of the α-globin transcription factor CP2. *J Biol Chem* 269:11663–11671.
13. Hansen U, Owens L, Saxena UH (2009) Transcription factors LSF and E2Fs: Tandem cyclists driving G0 to S? *Cell Cycle* 8:2146–2151.
14. Saxena UH, et al. (2009) Phosphorylation by cyclin C/cyclin-dependent kinase 2 following mitogenic stimulation of murine fibroblasts inhibits transcriptional activity of LSF during G1 progression. *Mol Cell Biol* 29:2335–2345.
15. Powell CMH, Rudge TL, Zhu Q, Johnson LF, Hansen U (2000) Inhibition of the mammalian transcription factor LSF induces S-phase-dependent apoptosis by down-regulating thymidylate synthase expression. *EMBO J* 19:4665–4675.
16. Bruni P, et al. (2002) Fe65, a ligand of the Alzheimer's β-amyloid precursor protein, blocks cell cycle progression by down-regulating thymidylate synthase expression. *J Biol Chem* 277:35481–35488.
17. Yoo BK, et al. (2009) Astrocyte elevated gene-1 regulates hepatocellular carcinoma development and progression. *J Clin Invest* 119:465–477.
18. Koehler AN (2010) A complex task? Direct modulation of transcription factors with small molecules. *Curr Opin Chem Biol* 14:331–340.
19. Venkatesan K, McManus HR, Mello CC, Smith TF, Hansen U (2003) Functional conservation between members of an ancient duplicated transcription factor family, LSF/Grainyhead. *Nucleic Acids Res* 31:4304–4316.
20. Kokoszynska K, Ostrowski J, Rychlewski L, Wyrwicz LS (2008) The fold recognition of CP2 transcription factors gives new insights into the function and evolution of tumor suppressor protein p53. *Cell Cycle* 7:2907–2915.
21. Zhong F, Swendeman SL, Popik W, Pitha PM, Sheffery M (1994) Evidence that levels of the dimeric cellular transcription factor CP2 play little role in the activation of the HIV-1 long terminal repeat in vivo or following superinfection with herpes simplex virus type 1. *J Biol Chem* 269:21269–21276.
22. Shirra MK, Hansen U (1998) LSF and NTF-1 share a conserved DNA recognition motif yet require different oligomerization states to form a stable protein-DNA complex. *J Biol Chem* 273:19260–19268.
23. Murata T, Nitta M, Yasuda K (1998) Transcription factor CP2 is essential for lens-specific expression of the chicken alphaA-crystallin gene. *Genes Cells* 3:443–457.
24. Li K, Foresee LN, Tunge JA (2005) Trifluoroacetic acid-mediated hydroarylation: Synthesis of dihydrocoumarins and dihydroquinolones. *J Org Chem* 70:2881–2883.
25. Vassilev LT, et al. (2004) In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303:844–848.
26. Haigh JA, Pickup BT, Grant JA, Nicholls A (2005) Small molecule shape-fingerprints. *J Chem Inf Model* 45:673–684.
27. Palli SR, Kapitskaya MZ, Kumar MB, Cress DE (2003) Improved ecdysone receptor-based inducible gene regulation system. *Eur J Biochem* 270:1308–1315.
28. Veljkovic J (2004) Role of the mammalian transcription factor LSF (late SV40 factor) in repression of DNA polymerase α gene expression. PhD dissertation (Boston University, Boston).
29. Cacioppo R (2008) Protein phosphatase 2A regulates phosphorylation of the transcription factor LSF in a cell growth-dependent manner. PhD dissertation (Boston University, Boston).
30. Rishi V, et al. (2005) A high-throughput fluorescence-anisotropy screen that identifies small molecule inhibitors of the DNA binding of B-ZIP transcription factors. *Anal Biochem* 340:259–271.
31. Waki K, et al. (2010) Establishment of functional telomerase immortalized human hepatocytes and a hepatic stellate cell line for telomere-targeting anticancer drug development. *Cancer Sci* 101:1678–1685.
32. Severgnini M, et al. (2011) A rapid two-step method for isolation of functional primary mouse hepatocytes: Cell characterization and asialoglycoprotein receptor based assay development. *Cytotechnology* 64:187–195.
33. Shea CM, Tzertzinis G (2010) Controlled expression of functional miR-122 with a ligand inducible expression system. *BMC Biotechnol*, 10.1186/1472-6750-10-76.
34. Drouin EE, Schrader CE, Stavnezer J, Hansen U (2002) The ubiquitously expressed DNA-binding protein late SV40 factor binds Ig switch regions and represses class switching to IgA. *J Immunol* 168:2847–2856.