Recognition of *c-ros* Oncogene Inhibition with a FRET-based Reporter Construct

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7.18 Topics in Experimental Biology

Nonscientific Abstract

Most cancers are caused by a variety of genes that control cell signaling and development. One of these genes is *c-ros*. Mutation turns on the *c-ros* gene, causing deadly types of brain cancer. We have engineered a protein that recognizes *c-ros* activity and that tests different chemical compounds to identify whether they turn off the *c-ros* gene. If a compound is found that turns off *c-ros* activity, it could be used as a drug to treat brain cancer.

Scientific Abstract

The *c-ros* oncogene is a receptor tyrosine kinase that has been found to be constitutively active in glioblastomas. To assay for active *c-ros*, we have designed a reporter construct that recognizes the phosphorylated state of the *c-ros* substrates. The reporter construct contains six parts: CFP, a *c-ros* substrate domain, a flexible linker, a recognition domain for tyrosine phosphorylation, YFP, and a nuclear export sequence. The construct changes conformation when *c-ros* phosphorylates the substrate domain, and this alteration can be recognized by measuring the Fluorescence Resonance Energy Transfer (FRET) between the CFP and YFP fluorophores. Efforts to engineer the construct are ongoing, and our preliminary results indicate successful insertion of the two fluorophores into the construct. After the reporter construct is fully engineered, we will use it to potentially identify specific chemical compounds that inhibit *c-ros* activity. This assay could lead to the discovery of a drug for the treatment of glioblastoma.

I. Specific Aims

Glioblastoma multiforme, one of the most malignant forms of neuro-ectodermal tumors, has repeatedly failed to respond to traditional drugs and treatments for cancer.[1,2] Therefore, new drugs and therapies are necessary to fight this disease. To stop the growth of glioblastoma multiforme tumors, we will search for an inhibitor of the *c-ros* protooncogene, which is constitutively expressed in 70 percent of patients with glioblastoma multiforme.[3,4,5,6,7,8] We will recognize inhibitors of *c-ros* using an engineered reporter construct that changes conformation when *c-ros* becomes inactive. The change in the conformation will be visualized by measuring the transfer of energy between the two fluorophores in the construct. This construct will be used to identify chemical compounds that may stop the growth of glioblastoma multiforme tumors by inhibiting the *c-ros* protooncogene.

Our specific aims are the following:

I. Assay development: To engineer a reporter construct that allows us to detect c-ros phosphorylation of its substrate.

II. Assay implementation: To use our engineered reporter construct to find a chemical compound that will inhibit the c-ros protooncogene.

II. Background and Significance

Glioma, a type of neuro-ectodermal tumor, is one of the most common and harmful tumors of the central nervous system. As mentioned earlier, the most malignant form of glioma is glioblastoma multiforme, which accounts for 50 to 60 percent of all cases of primary brain tumors and has poor prognosis with a life expectancy of less than three years.[5,6,9] Treatment of glioblastoma multiforme with chemotherapy, radiation, and aggressive surgery has been futile, thus necessitating other techniques and drugs to fight these tumors.[1,2]

Involvement of *C-ros* in Cancer

One gene that is mutated in a variety of gliomas is *c-ros*. This gene was originally identified in the UR2 avian sarcoma virus.[10,11] It was mapped to an area of chromosome 6q22 that contains mutations in protooncogenes like *c-myb* and *mas1*.[12,13,14,15] The colinkage of *c-ros*, *myb*, and *mas1* led researchers to believe that *c-ros* could also be involved in cancer.

Little work has been done on *c-ros* to date, but much of the conducted research focuses on how *c-ros* acts as a protooncogene. The early studies of homology, localization, and function of *c-ros* have all led to, or developed from the idea that *c-ros* is oncogenic when mutated.[16,17,18,19,20,21,22,23] Recently, tumor-forming mutations of *c-ros* have been studied for the fusions they form with other proteins. In the UR2 DNA tumor virus, oncogenic *c-ros* fuses to the p19 Gag protein. Unlike wild-type *c-ros*, this fusion oncogene does not dimerize.[24,25] In addition, an oncogenic version of *c-ros*, which results from a short intra-chromosomal deletion, fuses a novel Golgi apparatus-localized protein called Fig.[7,8] These observations support the theory that *c-ros* is involved in cancer formation.

Cancer studies consistently find *c-ros* to be active in glioblastomas and meningiomas.[3,4,5,6] In one study, 70 percent of mice with glioblastomas constitutively expressed the *ros* protein.[7,8] Because of this high incidence of oncogenic *c-ros* in glioblastomas, we have chosen to focus our research on inhibiting *c-ros* to stop the growth of glioblastomas.

***C-ros* as a Receptor Tyrosine Kinase**

The protooncogene *c-ros* is a type of receptor tyrosine kinase (RTK). RTKs function as vital signal transductors and often play a role in cell division or development. As a result, mutations in RTKs can result in unregulated cell growth and tumor formation.

All RTKs have similar mechanisms for activating signaling pathways. When a ligand binds to an RTK subunit, two RTK subunits dimerize and autophosphorylate each other on tyrosine residues.[26,27] A substrate for the phosphotyrosine domains binds to the tyrosine residues, and the dimerized RTKs phosphorylate the substrate on its tyrosine domain as well. Another molecule then binds to the phosphorylated substrate. This molecule is often a Src homology-2 domain (SH2) such as Src homology-2 phosphatase-2 (SHP2). The binding of an SH2 molecule to the substrate initiates a series of signals, which are used to control cell growth.[28]

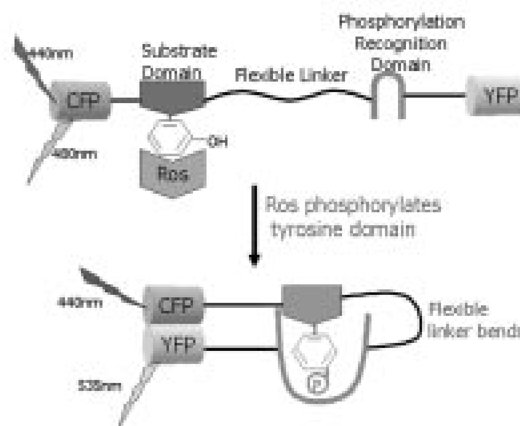


Figure 1. Construct design and FRET signals. The construct consists of five main fragments: CFP; a substrate domain for *c-ros* that contains a tyrosine phosphorylation site; a flexible linker; a recognition domain for tyrosine phosphorylation; and YFP. The conformation of the top construct occurs when *c-ros* has not phosphorylated its substrate. In this case, FRET emission at 440nm (high energy) will excite CFP, and CFP will emit light at 480nm, a slightly lower energy. When *c-ros* is active, it phosphorylates its substrate, resulting in a conformation change in the construct (bottom). The phosphorylation recognition domain binds to the phosphorylated substrate, bringing CFP and YFP into contact. The light emission from the fluorophores has now changed because some of the energy passes from CFP into YFP, exciting electrons in YFP and emitting out of YFP at 535nm.

Reporter Construct Recognition of Active *C-ros*

To determine whether the *c-ros* RTK is active and has phosphorylated its substrate, we have designed a reporter construct that will change conformation when the *c-ros* substrate has been phosphorylated. We based our design on previous reporter constructs, particularly the construct described in Sato et al.[29] Their reporter construct was developed as a method for visualizing protein phosphorylation by the insulin receptor. The construct contained six parts: Cyan Fluorescent Protein (CFP); a substrate domain of the insulin receptor that contains a tyrosine residue for phosphorylation by the receptor; a flexible linker; a recognition domain of tyrosine phosphorylation; Yellow Fluorescent Protein (YFP); and a nuclear export sequence (NES)[29] (Figure 1).

With the aid of fluorescence, the Sato reporter construct determined the activity of the insulin receptor. When the receptor was inactive, as indicated by its unphosphorylated substrate, the construct separated CFP and YFP, the two fluorophores in the construct. Therefore, when CFP, the donor molecule, was excited, the intensity from CFP was high. However, when the insulin receptor phosphorylated the substrate domain, the recognition domain for tyrosine phosphorylation bound to the tyrosine domain on the substrate, bringing YFP and CFP closer together. When the donor and accep-

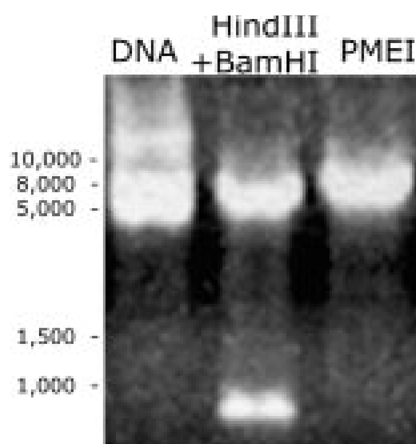


Figure 2. *[Preliminary Results]* Restriction digest of CFP-pcDNA3.1A. 1μg of DNA was run on a 1% agarose gel, and lane samples were stained with Ethidium Bromide. Lane 1 contains undigested CFP-pcDNA3.1A DNA; Lane 2 has DNA double digested with HindIII and BamHI; and Lane 3 contains DNA digested with PMEI. The double digest produced two bands, one for the CFP insert (700 bp) and one for the pcDNA3.1A vector (5.5 kb), producing a total of 6.2 kb.

tor fluorophores were in close proximity, fluorescence resonance energy transfer (FRET), characterized by the transfer of energy between fluorophores, occurred between CFP and YFP. This transfer resulted in a lower intensity from CFP and a higher intensity from YFP[30,31,32] (Figure 1).

The efficiency of FRET between the donor and acceptor fluorophores is given by the formula $1/\{1 + (R/R_o)6\}$, where R is the distance between the fluorophores and R_o is the distance at which half of the energy is transferred. The distance R between the two fluorophores can be determined after calculating R_o.[33] However, measurement of FRET between fluorophores is not entirely accurate. Therefore, the FRET technique is better suited for detecting relative changes in distance between the fluorophores rather than determining absolute distances. Sato et al. measured the change in the ratio of the fluorescence intensities of the fluorophores. As a result, measuring the FRET between CFP and YFP in the Sato construct is a valid way of determining the proximity of CFP to YFP. Researchers easily visualized the change in construct conformation between the phosphorylated and unphosphorylated states of the insulin receptor substrate, thus tracking the phosphorylation by the insulin receptor.

We chose to use this FRET-based reporter construct as a model for our own construct because we also want to recognize protein phosphorylation by a receptor. Using a similar construct, designed specifically for the *c-ros* protooncogene, phosphorylation of the *c-ros* substrate will result in a construct conformation change. With the aid of FRET measurement between the fluorophores, we will visualize whether *c-ros* has phosphorylated its substrate, and thus determine the activity of *c-ros*.

With this construct and the ability to tell if *c-ros* is active or inactive, inhibitors of *c-ros* can be discovered. Screening libraries of different chemical compounds on our construct for the changes induced in FRET will enable us to potentially discover a specific chemical inhibitor of the *c-ros* protooncogene.

III. Preliminary Results

In our preliminary experiments, we have successfully engineered part of the reporter construct, which we will use to assay *c-ros* activity. The reporter construct contains six parts: CFP; a substrate of *c-ros* containing a tyrosine domain; a flexible linker; a recognition domain for tyrosine phosphorylation; YFP; and a NES.[29] We successfully subcloned the CFP and YFP segments into a pcDNA3.1A plasmid (Invitrogen) and transformed the recombinant DNA into DH5α cells, a cell line derived from *Escherichia coli*. Each remaining segment of the construct will be subcloned individually into the plasmid using restriction digests and ligations.

We initially subcloned CFP into pcDNA3.1A. After the insert and vector ligated correctly, the DNA was transformed into DH5α cells. After plating and inoculating the colonies, minipreps were used to isolate DNA from the colonies (see Research Design). 1μg of the DNA from each of the minipreps was digested with restriction enzymes to confirm presence of the CFP insert in the pcDNA3.1A vector, and the digests were run on a 1 percent agarose gel (Figure 2). The double digest of our pcDNA3.1A-CFP plasmid with XbaI and PME produced two bands, one for our CFP insert (~ 700 bp) and one for the vector (~ 5.5 kb). Sequencing the DNA from the minipreps confirmed that the subcloning was successful and that no mutations had developed in our plasmid (MGH DNA Core Facility; data not shown).

YFP was then inserted into the pcDNA3.1A-CFP plasmid, resulting in the successful assembly of the CFP and YFP fragments of our construct. Subcloning of YFP will be followed by insertion of the remaining sequences in the following order: a NES, a flexible linker, a substrate

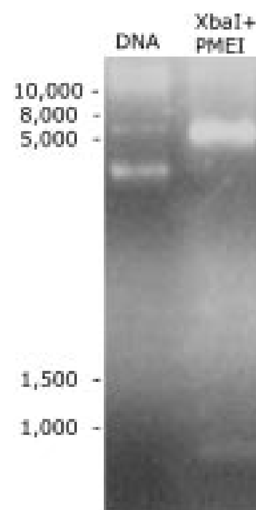


Figure 3. Restriction digest of CFP-YFP-pcDNA3.1A. 1μg of DNA was run on a 1% agarose gel and lane samples were stained with Ethidium Bromide. Lane 1 contains undigested CFP-YFP-pcDNA3.1A DNA and Lane 2 has DNA digested with XbaI and PMEI. The double digest produced two bands, one for the YFP insert (700 bp) and one for the CFP-pcDNA3.1A vector (6.2 kb), producing a total of 6.9 kb.

domain for *c-ros*, and a recognition domain for tyrosine phosphorylation. Each of these segments will be similarly subcloned to CFP. A final restriction digest will always be used to confirm the insertion of each major segment into the plasmid (Figure 3). After each confirmation digest and gel electrophoresis, DNA sequencing will verify the correct arrangement of our recombinant plasmid, as it did for insertion of CFP and YFP into pcDNA3.1A.

IV. Research Design

The goal of our research design is to efficiently achieve Specific Aims I and II. We plan to implement an assay to screen different chemical compounds for the ability to inhibit oncogenic *c-ros*. The assay requires engineering a reporter construct in which FRET is used to determine the activity of *c-ros*. Our assay provides a simple way of screening for inhibitors of *c-ros* by applying a chemical compound to a cell with active *c-ros* and measuring the FRET between CFP and YFP. Using this method, we will visualize whether the phosphorylated substrate domain becomes dephosphorylated. If a compound results in dephosphorylation of the *c-ros* substrate, it is able to inactivate the *c-ros* protooncogene.

Specific Aim I: Assay Development

As discussed in the Preliminary Results, we are working on pursuing Specific Aim I by engineering a reporter construct to recognize when the *c-ros* protooncogene phosphorylates its substrate. This construct has six individual segments. The cDNAs for these fragments will be generated by standard PCR and subcloned into the restriction sites of plasmid pcDNA3.1A (Invitrogen) (Figure 4A).

The six fragments of our reporter construct will be subcloned individually into pcDNA3.1A. Initially, cDNA for each fragment will be amplified by PCR using primers that incorporate flanking restriction sites into the ends of the molecules. The sequences for these primers are given in Table 1. Both the cDNAs and plasmid will then be digested with appropriate restriction enzymes to allow for proper ligation (Figure 4B). Restriction digests will follow standard protocols.[34] We will ligate the vectors and inserts using T4 DNA Ligase and then transform the recombined plasmids into DH5α cells. Colonies will be grown on LB Agar plates with Carbenicillin in order to select for transformed colonies with Carbenicillin resistance. Minipreps will then be used to isolate the DNA from the colonies. We will sequence these DNAs after insertion of every construct fragment to confirm that each subcloning is successful and that no mutations develop in the molecules. Insertion of a nuclear export sequence into the construct will ensure that the construct can be exported from the nucleus to the cytoplasm (Table 1). Assembly of the construct achieves Specific Aim I.

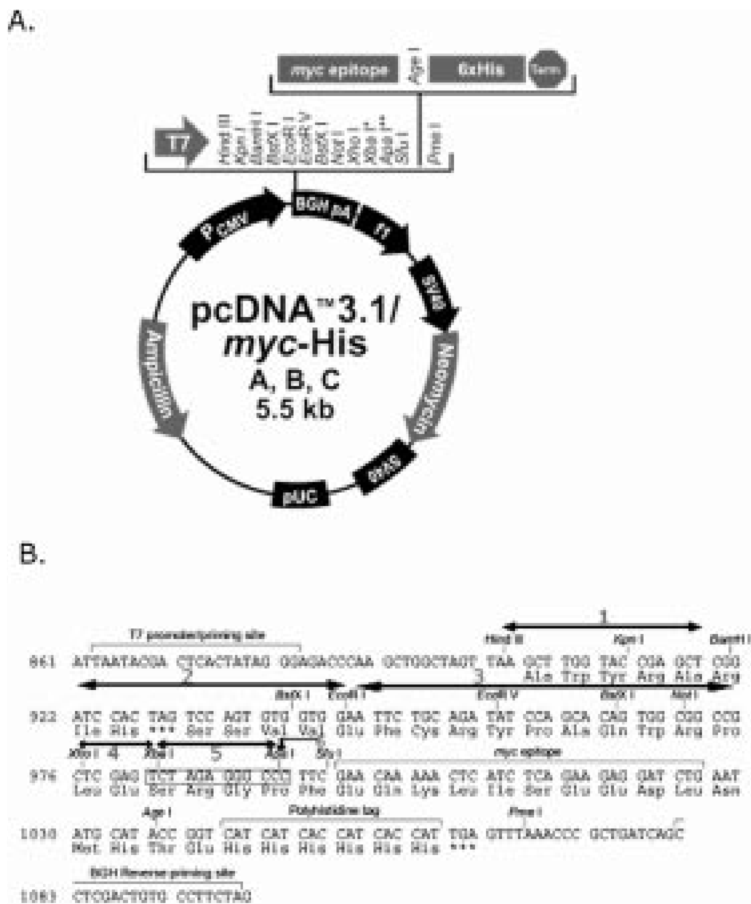


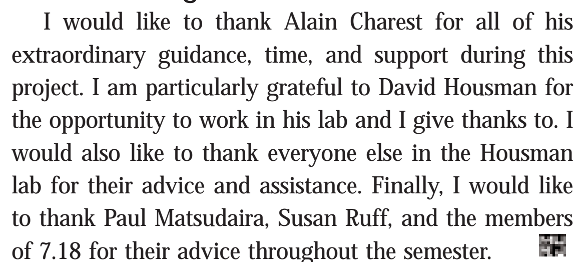
Figure 4. Map and sequence of plasmid pcDNA3.1A. A: We subcloned our construct into pcDNA3.1A using the restriction enzyme sites listed. B: Sequence of multiple cloning site of pcDNA3.1A, which indicates where our construct was inserted. The red numbers indicate the position of insertion for each fragment of the construct. (1) CFP was inserted between the HindIII and BamHI sites; (2) substrate domain between BamHI and EcorI sites; (3) flexible linker between EcorI and XhoI sites; (4) recognition domain of tyrosine phosphorylation between XhoI and XbaI sites; (5) YFP between the XbaI and ApaI sites; (6) and the NES immediately after YFP at the ApaI site.

Specific Aim II: Assay Implementation

Inhibitors of *c-ros* can be discovered by using our construct, which is Specific Aim II. After engineering our construct, we will transfect it into the mammalian cell line 293T. These cells have an active *c-ros* protooncogene, so intracellular *c-ros* will interact with the construct. The phosphorylation of the substrate domain in our compound by active *c-ros* will change the conformation of our construct, similar to the construct discussed in Sato et al. (see Background and Significance). We will measure the FRET between the two fluorophores, CFP and YFP, to determine when the *c-ros* substrate is phosphorylated (see Background and Significance; Figure 1). Screening for FRET between the fluorophores

PRIMER/SEQUENCE NAME	SEQUENCE (5' to 3')
HindIII-CFP	AAAAAAAAGCTTCCACCATGGTGAGCAAGGGCGAGGAGCTGTTC
BamHI-CFP	ATCACTCTCGGCATGGACGAGCTGTACAAGGGATCCAAAAAA
XbaI-YFP	AAAAAATCTAGAGTGAGCAAGGGCGAGGAGCTGTTCACC
ApaI-YFP	ACTCTCGGCATGGACGAGCTGTACAAGGGGCCCAAAAAA
Linker-10	GNNGNNGGSNNGGNNGNGG
Linker-20	GNNGGNGGSNNGNGGNGNNGGNNGNGG
NES	AAAAAAGGGCCCTTACCACCATTAGAGAGATTAACGTTATAGTGAGGGCCCAAAAAA

Table 1. Sequences for PCR primers and parts of the construct. PCR primers for adding restriction sites and amplifying CFP and YFP are shown. Sequences for the flexible linker and nuclear export sequence (NES) are also given.



in mammalian cells enables us to test for a construct in which *c-ros* is actively phosphorylating its substrate.

After measuring the FRET between the fluorophores in cells with an active *c-ros*, we will put varying concentrations of different chemical compounds on the cells. We will then measure the FRET between the fluorescent proteins of the construct. Changes in FRET between the fluorophores after application of a chemical compound indicate that the substrate was recently dephosphorylated and that the chemical compound inhibited the function of the protooncogene. The discovery of a chemical compound that inhibits the *c-ros* protooncogene fulfills Specific Aim II, and we plan to achieve this goal within the next five years.

Conclusion

In conclusion, we plan to design and implement an assay to screen different chemical compounds for their ability to inhibit oncogenic *c-ros*. This assay will allow for simple screening of *c-ros* inhibitors using our FRET-based reporter construct. Consequently, our engineered receptor construct and assay could potentially result in the discovery of chemical compounds that would serve as effective drugs against cancers induced by constitutively active *c-ros*, such as glioblastoma multiforme. This work can also be further expanded to facilitate the discovery of inhibitors of other receptor tyrosine kinases. Thus, this assay may result in effective drugs for a variety of cancers in the near future.

V. Acknowledgments

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References

1. McLendon, R.E., and E.C. Halperin. (2003). Is the long-term survival of patients with glioblastoma multiforme overstated? Cancer 98: 1745–1748.
2. Fiveash, J.B., and S.A. Spencer. (2003). Role of radiation therapy and radiosurgery in glioblastoma multiforme. Cancer 9: 222–229.
3. Birchmeier, C., S. Sharma, and M. Wigler. (1987). Expression and rearrangement of the *c-ros* gene in human glioblastoma cells. Proc. Natl. Acad. Sci. USA. 84: 9270–9274.
4. Sharma, S., et al. (1989). Characterization of the *c-ros*-gene products exrpressed in human glioblastoma cell lines. Oncogene Res. 5: 91–100.
5. Watkins, D., F. Dion, M. Poisson, J. Delattre, and G. Rouleau. (1994). Analysis of oncogene expression in primary human gliomas: evidence for increased expression of the *ros* oncogene. Cancer Genet. Cytogenet. 72: 130–136.
6. Zhao, J., and S. Sharma. (1995). Expression of the *c-ros* oncogene for tyrosine receptor kinase in adult human meningiomas. Cancer Genet. Cytogenet. 83: 148–154.
7. Charest, A., et al. (2003). Oncogenic targeting of an activated tyrosine kinase to the Golgi apparatus in a glioblastoma. Proc. Natl. Acad. Sci. USA. 100: 916–921.
8. Charest, A., et al. (2003). Fusion of FIG to the receptor tyrosine kinase ROS in a glioblastoma with an interstitial del(6)(q21q21). Genes Chr. Cancer. 37: 58–71.
9. Wu, J., and D. Chikaraishi. (1990). Differential expression of *ros* oncogene in primary human astrocytomas and astrocytoma cell lines. Cancer Res. 50: 3032–3035.
10. Neckameyer, W.S., and L.H. Wang. (1985). Nucleotide sequence of avian sarcoma virus UR2 and comparison of its transforming gene with other members of the tyrosine protein kinase oncogene family. J. Virol. 53: 879–884.
11. Matsushime, H., L. Wang, and M. Shibuya. (1986). Human *c-ros-1* gene homologous to the *v-ros* sequence of UR2 sarcoma virus encodes for a transmembrane receptorlike molecule. Mol. and Cell Biology 6: 3000–30004.
12. Nagarajan, L., et al. (1986). The human *c-ros* gene (*ros*) is located at chromosome region 6q16-6q22. Proc. Natl. Acad. Sci. USA. 83: 6568–6572.
13. Satoh, H., M. Yoshida, H. Matsushime, M. Shibuya, and M. Sasaki. (1987). Regional localization of the human *c-ros-1* on 6q22 and *flt* on 13q12. Jpn. J. Cancer Res. 78: 772–775.
14. Rabin, M., et al. (1987). Human *c-ros* and *mas1* oncogenes located in regions

of chromosome 6 associated with tumor-specific rearrangements. Oncogene Res. 1: 169–178.

15. Hubbard-Smith, et al. (1992). Altered chromosome 6 in immortal human fibroblasts. Mol. and Cell Biology 12: 2273–2281.
16. Podell, S.B. and B.M. Sefton. (1987). Chicken protooncogene *c-ros* cDNA clones: identification of a *c-ros* RNA transcript and deduction of the amino acid sequence of the carboxyl terminus of the *c-ros* product. Oncogene 2: 9–14.
17. Matsushime, H., and M. Shibuya. (1990). Tissue-specific expression of rat *c-c-ros* gene and partial structural similarity of its predicted products with sev protein of Drosophila melanogaster. J. Virol. 64: 2117–2125.
18. Chen, J., D. Heller, B. Poon, L. Kang, and L. Wang. (1991). The protooncogene *c-ros* codes for a transmembrane tyrosine protein kinase sharing sequence and structural homology with *sevenless* protein of *Drosophila melanogaster*. Oncogene. 6: 257–264.
19. Narayana, L, and L. Nagarajan. (1992). A mouse *c-ros* genomic clone: identification of a highly conserved 22-amino acid sequence in the juxtamembrane domain. Gene 118: 297–298.
20. Birchmeier, C., E. Sonnenberg, K.M. Weidner, and B. Walter. (1993). Tyrosine kinase receptors in the control of epithelial growth and morphogenesis during development. Bioessays 15: 185–190.
21. Chen, J., C. Zong, and L. Wang. (1994). Tissue and epithelial cell-specific expression of chicken protooncogene *c-ros* in several organs suggests that it may play roles in their development and mature functions. Oncogene. 9: 775–780.
22. Kanwar, Y.S., Z. Liu, A. Kumar, J. Wada, and F. Carone. (1995). Cloning of mouse *c-ros* renal cDNA, its role in development and relationship to extracellular matrix glycoprotiens. Kidney International 48: 1646–1659.
23. Sonnenberg-Riethmacher, E., B. Walter, D. Riethmacher, S. Godecke, and C. Birchmeier. (1996). The *c-ros* tyrosine kinase receptor controls regionalization and differentiation of epithelial cells in the epididymis. Genes Dev. 10: 1184–1193.
24. Chan, J.L.K, M. Lai, and L. Wang. (1997). Effect of Dimerization on Signal Transduction and Biological Function of Oncogenic Ros, Insulin, and Insulin-like Growth Factor I Receptors. J. Biol. Chem. 272: 146–153.
25. Zong, C.S., J.L.K. Chan, S.K. Yang, and L.H. Wang. (1997). Mutations of *ros* differentially affecting signal transductions pathways leading to cell growth versus transformation. J. Biol. Chem. 272: 1500–1506.
26. Yu, K., and M. Czech. (1984). Tyrosine phosphorylation of the insulin receptor beta subunit activates the receptor-associated tyrosine kinase activity. J. Biol. Chem. 259: 5277–5286.
27. Haring, H., et al. (1985). Interaction of the insulin receptor kinase with serine/threonine kinases in vitro. J. Biol. Chem. 28: 171–182.
28. DeClue, J.E., I. Sadowski, G.S. Martin, and T. Pawson. (1987). A conserved domain regulates interactions of the v-fps protein-tyrosine kinase with the host cell. Proc. Natl. Acad. Sci. USA. 84: 9064–9068.
29. Sato, M., T. Ozawa, K. Inukai, T. Asano, and Y. Umezawa. (2002). Fluorescent indicators for imaging protein phosphorylation in single living cells. Nature Biotech. 20: 287–294.
30. Morris, S.J., T.C. Sudhof, and D.H. Haynes. (1982). Calcium-promoted energy resonance transfer between fluorescently labeled proteins during aggregation of chromaffin granule membranes. Biochem. Biophys. Acta. 693: 425–436.
31. Chantler, P.D., T. Tao, and W.F. Stafford, III. (1992). On the relationship between distance information derived from cross-linking and from resonance energy transfer, with specific reference to sites located on the myosin head. Biophys. J. 59: 1242–1250.
32. Yang, H., et al. (2003). Protein conformational dynamics probed by single-molecule electron transfer. Science 302: 262–266.
33. Stryer, L., and R. Haugland. (1967). Energy transfer: a spectroscopic ruler. Proc. Natl. Acad. Sci., USA. 58: 719–726.
34. Philippsen, P., Streeck, R.E., and Zachau, H.G. (1975). Investigation of the repetitive sequences in calf DNA by cleavage with restriction nucleases. Eur. J. Biochem. 57: 55–68.
35. Birchmeier, C., K. O'Neill, M. Riggs, and M. Wigler. (1990). Characterization of *c-ros* cDNA from a human glioblastoma cell line. Proc. Natl. Acad. Sci. USA. 87: 4799–4803.
36. Liu, Z.Z., et al. (1996). Comparative role of phosphotyrosine kinase domains of *c-ros* and *c-ret* protooncogenes in metanephric development with respect to growth factors and matrix morphogens. Dev. Bio. 178: 133–148.
37. Wagenfeld, A., C. Yeung, W. Lehnert, E. Nieschlag, and T.G. Cooper. (2002). Lack of glutamate transporter EAAC1 in the epididymis of infertile *c-ros* receptor tyrosine-kinase deficient mice. J. Andrology. 23: 772–782.