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13. ABSTRACT *(Maximum 200 Words)*

The present document summarizes the work of three years in which we have endeavored to better understand the mechanisms that lead to NF1 and the relationship between NF1 and the related rasGap gene. The approach was to generate conditional mutations in both the NF1 and rasGap genes as a means of combining these two otherwise embryonic lethal mutations to examine their cooperation in the physiology of cells, in disease and in tumor formation. While we succeeded in generating the NF conditional mutation, we failed to generate the rasGap mutation. However the study of conditional mutant mice for NF1 has yielded unexpectedly rewarding data. These mice have demonstrated their utility in teaching us about cancer, astrogliosis and many other features of NF1 that are seen in patients. The fruits of the work in the preceding funding period have generated a multiplicity of exciting projects that will be the subject of study in our laboratory and many other laboratories for years to come.

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The primary goal of this award has been to examine the role of NF1 and p120rasGap embryonic development as well as their interaction in NF1 disease. This work has moved forward at an accelerated pace in some areas and due to technical limitations at a relatively slower pace in others. The components of the proposal that rested on the generation and analysis of a conditional mutation at the NF1 gene have moved rapidly and already revealed a rich source of information. Below, I describe the progress in objective 1 as related to tasks 1-6. A hindrance in a component of the proposed work has been the difficulty in generating a conditional mutant of the p120Gap. Thus many of the objectives or on hold until this mutation is transmitted to the germ line. However, in lieu of the p120Gap mutation, Dr. Henkemeyer has made considerable progress (as outlined) in the understanding ofp120Gap signaling inside the cell and in other highly related areas of intracellular signaling and this progress will be very instructive in our analysis of subsequent experiments.

Objective 1. Tasks 1-2. (Parada)

Our previous experience with the generation of a null mutation has confirmed that exon 31 is essential for NF1 function (Brannan et al., 1994; Vogel et al., 1995). Figure 3A indicates the strategy we employed to place *lox* sites flanking exons 31 and 32. PCR primers encoding the *lox* sequences (34bp) plus an Eco R1 restriction enzyme - site that are designed for diagnostic purposes (18 bp) - were inserted into introns that flank exons 31 and 32 of the NF1 gene. In this construct, the neo selectable marker lies within the *lox* sites such that after *cre* mediated recombination, the neo gene is removed together with exons 31 and 32. ES cells were next transfected with the target vector and homologous recombinant cells were screened, clonally purified and injected into blastocysts for the formation of germline chimeric mice. Germline transmission was obtained and heterozygous (Figure 3B) and homozygous (Figure 3C) mice were viable as indicated by South ern blots employing probes external to the recombination

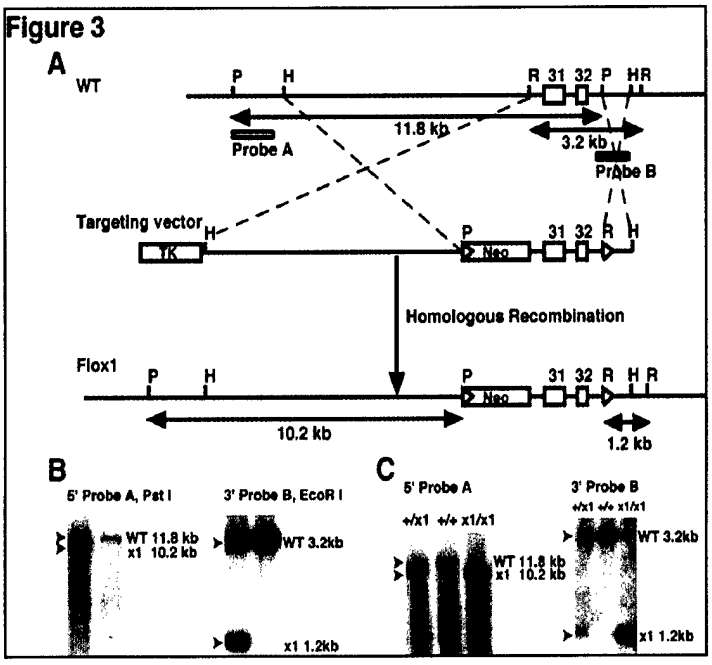
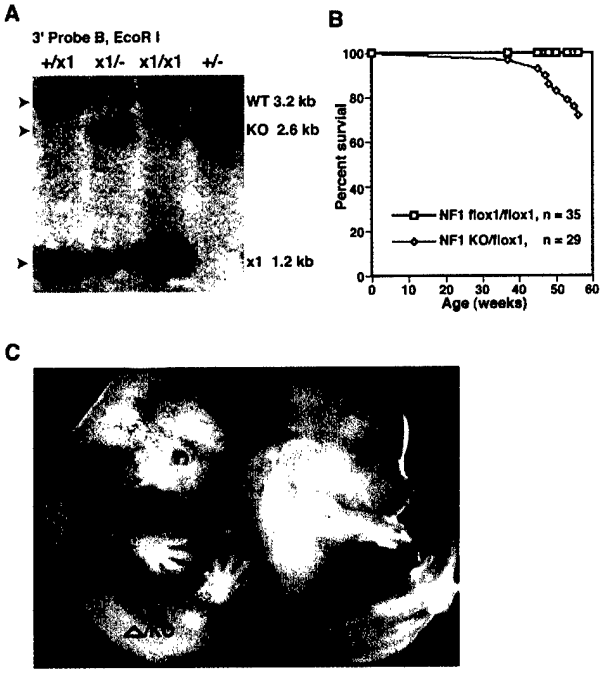


Figure 4



cassette. We next examined the viability of the recombinant (**NF1flox**) allele. This was done by crossing the conditional allele into the null background to generate compound heterozygous mice that had a null allele on one chromosome and the NF1flox allele on the second chromosome (Figure 4A; x1/-). As shown in Figure 4B, the **NF1flox/-** compound heterozygous mice behave indistinguishably from NF1+/- heterozygotes and the **flox/flox** homozygotes are indistinguishable from wildtype mice. As previously demonstrated, NF1+/- mice begin to die around 11 months. Thus by survival criteria, the NF1flox allele appears indistinguishable from the wild type allele in spite of harboring to flox sites and a neo gene within its intronic sequences.

The original NF1-/- null embryos perish at E13.5 and are characterized by edema, abnormal heart, vascular malfunction and reduced pigmentation of the retina (Brannan et al., 1994; Jacks et al., 1994; Vogel and Parada, 1998). We have crossed the NF1flox/- compound heterozygote to a transgenic Cre expressing mouse line that expresses the cre recombinase in the germline. Thus effective ablation of the NF1flox allele in the germline should result in a phenocopy of the original NF1-/- embryos. As shown in Figure 4C, NF1flox/-;TG-cre mice are indistinguishable from NF1-/- embryos. This demonstrates that *in vivo*, the NF1flox allele is readily recombined to make a null allele through coexpression of cre recombinase.

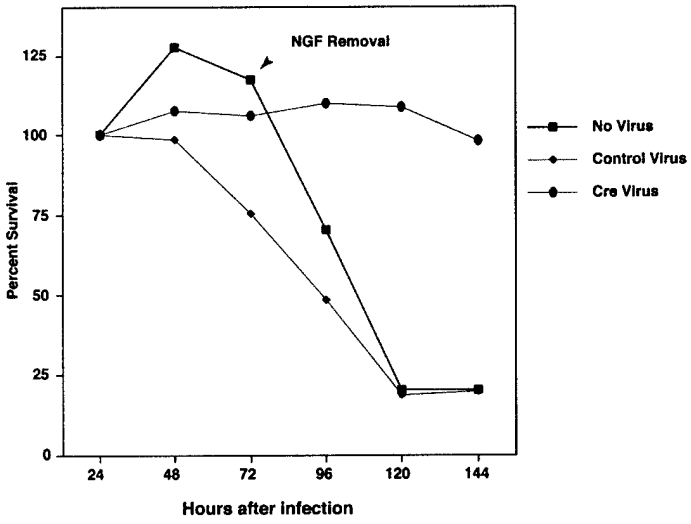
These results indicate the successful generation of a conditional mutation of the NF1 gene. The entirety of the present proposal will center on exploitation of this functional model system to explore NF1 function in vivo and in primary culture.

Tasks 3-4. Primary culture studies.

DRG neurons: In the previous award period, we demonstrated that DRG neurons lacking NF1 function were liberated of neurotrophin requirement for survival. Our interpretation of the data was that removal of NF1 activated the ras pathway even in the absence of neurotrophins thus rendering the neurons neurotrophin independent. This interpretation, though consistent with all the data, could not rule out the formal possibility that the loss of neurotrophin requirement by sensory neurons might not be the consequence of earlier developmental effects (perhaps in the neural crest) which could lead to the formation of neurotrophin independent neurons.

With the availability of the NF1flox mice we have finally been able to address this issue directly. As we have previously shown, DRG neurons can be cultured directly from mice and be exposed and productively infected by adenoviruses (Klesse and Parada, 1998). We have used adenoviral technology, which is well established in my laboratory, to produce a CMV-Cre adenovirus. NF1flox/- compound heterozygous embryonic DRG neurons to cre recombinase can be scored by PCR (not shown). At MOI=20, virtually

Figure 6

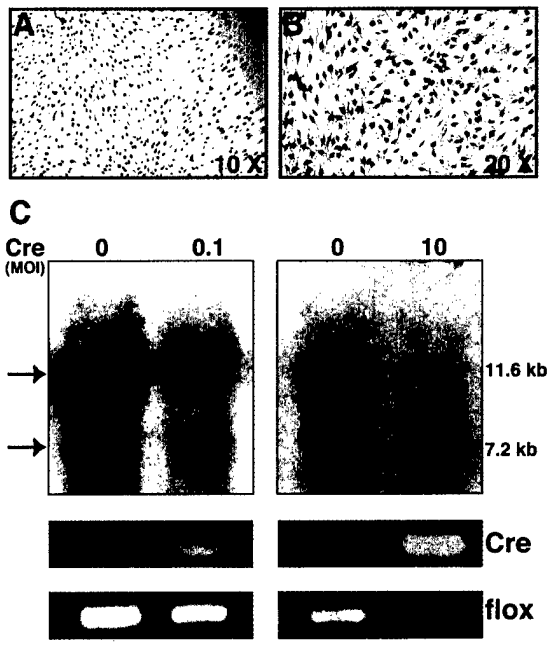


all DRG neurons in the cultures show deletion of the conditional allele.

Thus, DRG cultures can be effectively manipulated to ablate NF1 for cell biological and biochemical experiments. As indicated in Figure 6, neurons were cultured from NF1flox/- mice and subjected either to mock infection (no virus), infection with a CMV-lacZ expressing virus (Control virus), or with the CMV-cre virus. After neurotrophin removal, cultures were monitored for survival. Only the cultures exposed to Cre recombinase (and thus rendered NF1 deficient) survived in the absence of neurotrophins. These data provide firm evidence that the action of NF1 is directly on neurotrophin signalling. Specific Aim 1 will directly address and extend these studies to additional neuronal subtypes and to later times in development.

Schwann cells: Schwann cells or their precursors are likely the originators of neurofibromas and/or neurofibrosarcomas. Ratner and colleagues have made elegant studies to examine the properties of embryonic NF1-/-Schwann cells. However these studies have been hampered by the limitation that these cells can only be produced from a limited window of opportunity (before E13.5) and the quantities that are obtained are limited. We have cultured adult sciatic nerve Schwann cells from the NF1flox mice using standard culture procedures provided us by Mary Bunge. We are able to collect the sciatic nerves of a large number of mice at once and thus can prepare large quantities of cells. As shown in Figure 7, these cells grow robustly in the presence of forskolin and glial growth factor (GGF). Furthermore, these cells can be efficiently infected with adenoviruses. As shown in panels A & B, greater than 90% of the cells stain for Lac Z. Figure 7C shows Southern blot (upper) and PCR based (lower) assays for reduction of the gene by coexposure to the cre-expressing adenovirus. MOI =10 is sufficient to ablate NF1 from the majority of cells.

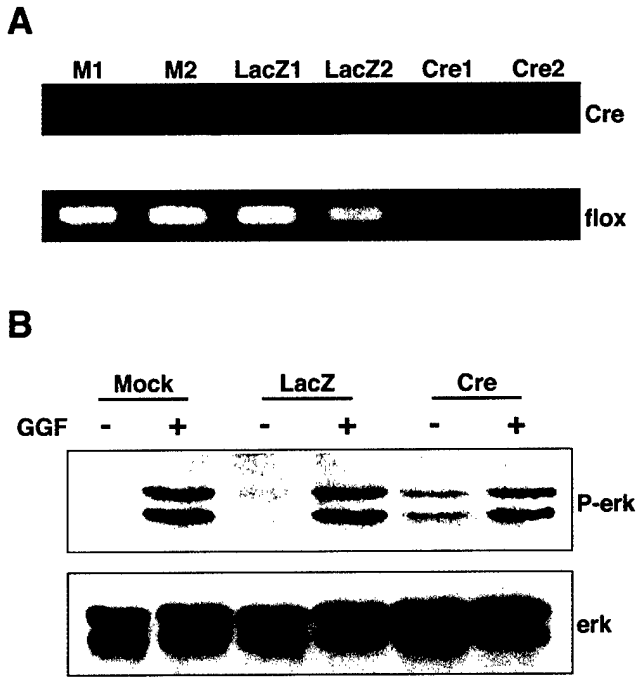
Figure 7



The ability to grow large numbers of cells will afford us the possibility of performing assays that are otherwise impractical with the original null NF1 embryos. As shown in Figure 8, NF1flox Schwann cells were either mock infected (M1 & M2), or exposed to LacZ or Cre expressing adenoviruses. Panel A shows that only the Cre infected cells recombined the floxed genes. These Schwann cell cultures were maintained in the presence or absence of GGF. As shown in Panel B, phopho-erk antibodies detect activated erk only in the GGF cultured cells except for the Cre recombined cells. In this latter case, erk is found active in the cultures that do not have GGF. These data demonstrate that downstream effectors of the ras pathway are constitutively activated in Schwann cells when NF1 function is removed.

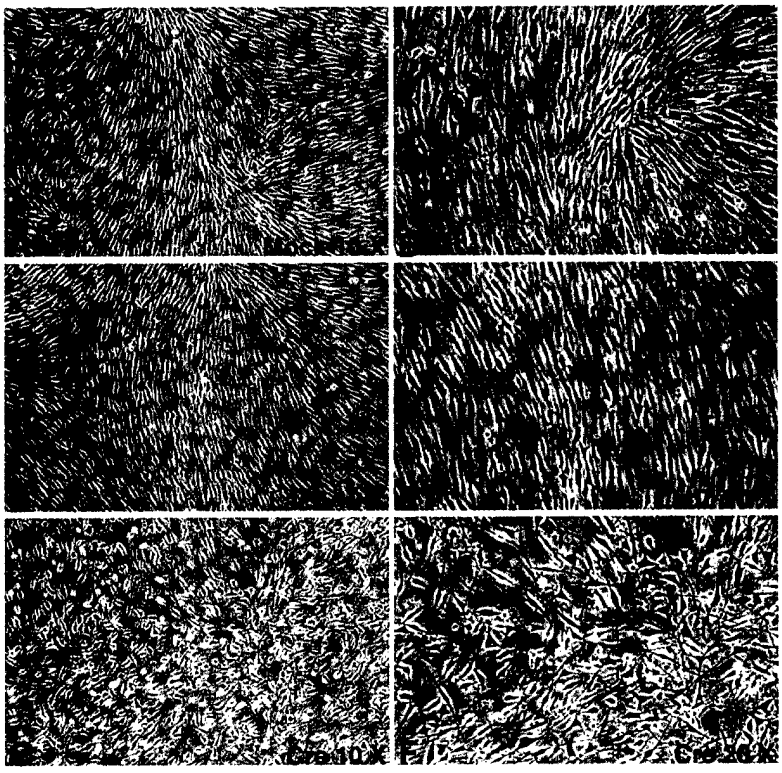
Cultured Schwann cells have a distinct morphology characterized by alignment into organized swirls (Figure 9 A-E). However, upon exposure to cre recombinase and ablation of NF1 function, these cells acquire a distinct morphological change. As seen in Figure 9 C & D, elimination of NF1 function causes the cells to become refractile and disorganized. By classical oncogenic criteria these cells have lost contact inhibition and appear morphologically transformed.

Figure 8



Task. 5 Promoter specific Cre Adenoviruses: As described in detail below, tissue specific promoters will be employed to drive Cre transgene expression for the purpose of inactivating NF1 in specific tissue compartments. An additional approach toward spatial and temporal inactivation of NF1 is through the application of Adenoviruses to cultures as described above, or *in vivo*. We have therefore undertaken to generate recombinant adenoviruses that express cre under neuronal or glial specific promoters. These viruses will be used to inactivate NF1 regionally in the NF1flox mouse.

Figure 9



Task 6. Synapsin I-cre transgenic mice. The Synapsin I gene is expressed exclusively in neurons. Our collaborator at UCSD, Dr. Jamey Marth, has provided us with two independent lines of cre-expressing transgenic mice under the control of the Synapsin I promoter (Syn-cre mice).

ß-actin/LacZ reporter mouse. While the description of tissue specific promoters has been in the literature for many years, it is evident that the use of such promoters to drive transgene expression, are not always successful. Introduction of transgenes into the genome can result in modulatory expression effects. The neighboring

chromatin can sometimes exert control over the introduced promoter and cause ectopic, circumscribed, or diminished expression. It is therefore important to characterize each transgenic founder to determine the precise expression in that mouse and its derivatives. Therefore, to ascertain the spatial and temporal expression of cre in the transgenic mice to be used in this proposal, we employ a reporter mouse strain that harbors a beta-actin driven LacZ gene that requires cre function for activation of the lacZ gene. Thus only tissues where cre has been expressed stain for LacZ. Figure 10 shows four panels of mouse embryos stained for lacZ. The Syn-cre transgenic (upper left) and the beta-actin reporter transgenic (upper right) do not stain blue. However, upon crossing these mice, the resultant progeny beta-actinLacZ/Syncre) show _-gal activity in tissues that normally express the Synapsin I gene, namely in differentiated neurons as evidenced by expression in the head and spinal cord region. Figure 11 shows vibratome sections of the Syn-cre/lacZ embryos. Figure 11, Panel A illustrates the absence of staining in E12.5 ventricular regions where undifferentiated neuronal progenitors are in the process of dividing. LacZ staining is limited to the peripheral regions of the embryonic brain where maturation and differentiation is taking place. Panel B shows LacZ expression in the spinal cord and lightly in DRG. A sagittal view of the thoracic region of an E15.5 embryo illustrates the cranio-caudal nature of embryonic development with the more mature cranial DRG expressing the strongest LacZ in a gradient toward the less differentiated, lighter staining ganglia more caudal.

Figure 10

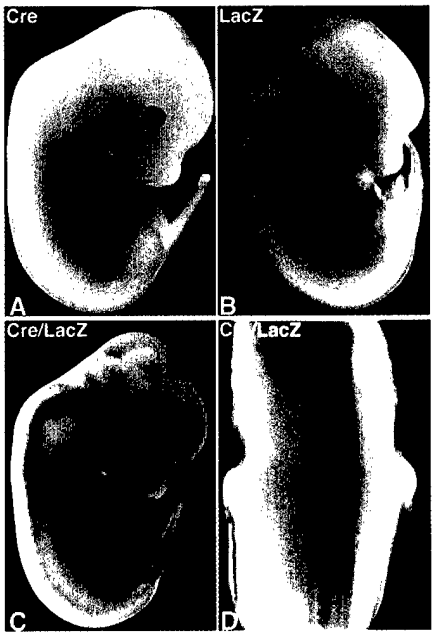
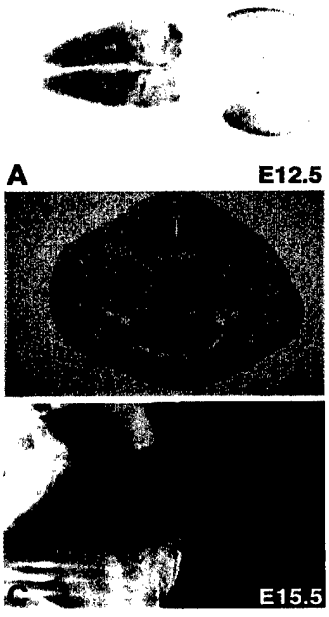


Figure 11



To further examine the specificity of Cre expression in the Syn-cre mouse, we examined histological sections of the CNS using immunohistochemistry with LacZ and neuron specific monoclonal (NeuN) antibodies (Chemicon International Inc.; MAB377). Figure 12 shows immunofluorescence of the neo-cortex (A-F) and spinal cord (G-I) of Syn-cre/LacZ newborn pups. Red fluorescence is a neuron specific staining and Green fluorescence is the

LacZ specific antibody (note that we observe some non-specific background staining with this

Figure 12

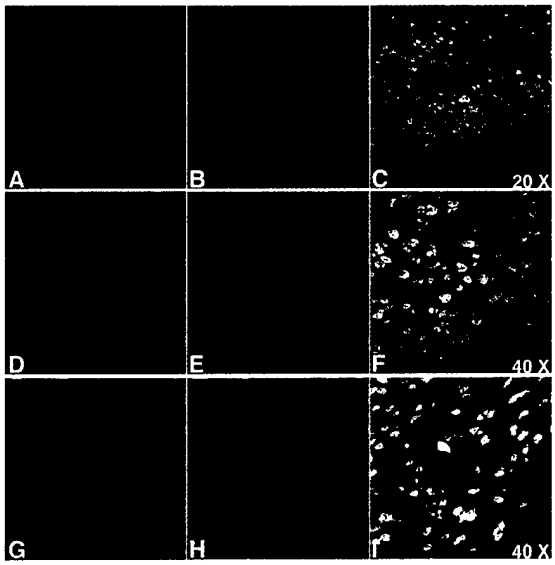
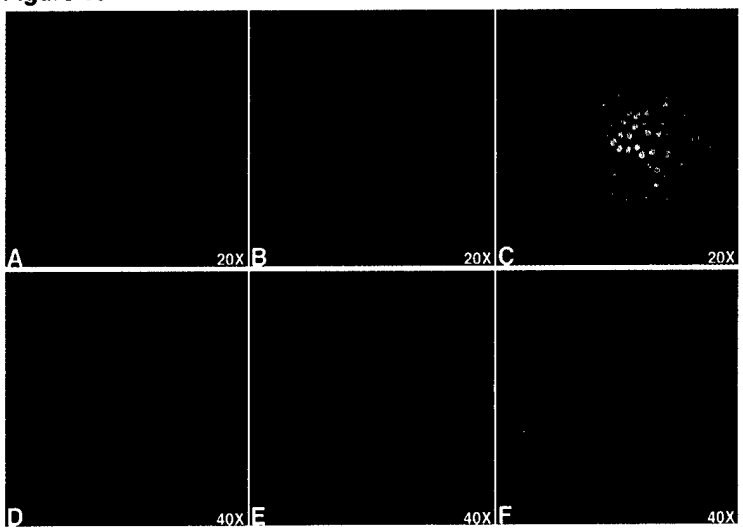


Figure 13



antibody). The right panels show double labelling in yellow with very accurate colabelling of the neuron specific and LacZ antibodies indicating cre activity specifically in neurons. Figure 13 examines coexpression of LacZ and neuron specific markers in the hippocampus (A-C) and cerebellum (D-F). In CA1-CA3 boundary of the hippocampus it is again clear that the LacZ activity is confined to neurons. Our NeuN monoclonal antibody is curiously not expressed in Purkinje cells (see manufacturer; Figure 13D). Thus, only the granule cell layer fluoresces red. The synapsin promoter however induced cre expression (and thus LacZ) in both Purkinje cells and in granule cells (Figure 4E). Thus, colabelling is confined to the granule layer even though lacZ staining remains evident in Purkinje cells.

Task 7. Analysis of Synapsin 1-cre mice.

It has been shown that brains from NF1 patients can have astrogliosis. Furthermore, NF1 heterozygous mice display moderately increased astrocytes in specific brain regions including hippocampus. This glial-specific phenotype seems context-dependent and non-cell autonomous, To test whether the astrogliosis seen in human and mouse brains may result from loss of NF1 in neurons, brains from adult control and mutant mice were serially sectioned and subjected to immunohistochemistry using the astrocyte specific anti-GFAP antibody. Ten of 10 $NF1^{SynIKO}$ mice displayed extensive astrogliosis in the entire brain. The intense GFAP staining was especially evident in the cerebral cortex where endogenous GFAP immunoreactivity is normally low. The GFAP-positive cell morphology to be typical of reactive astrocytes.

It has been proposed that learning deficits in NF1 heterozygous mice may be due to astrogliosis in the hippocampus. We therefore examined GFAP immunoreactivity in control and mutant hippocampus. The mutant hippocampus not only showed increased number of GFAP-positive astrocytes, but also displayed hypertrophy of astrocytes with thicker and increased processes. Other brain regions also showed dramatic increase in number of GFAP-positive astrocytes including brainstem since there is little endogenous GFAP immunoreactivity. To determine the degree of the increase in GFAP-positive astrocytes in the mutant brains, we quantified the number of GFAP-positive cells in cerebral cortex,

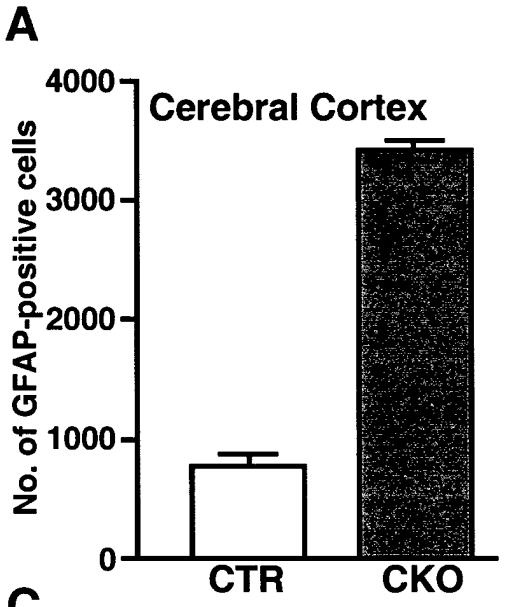
hippocampus and brainstem. As indicated in Table 1, there is 4.4 fold increase in the mutant cerebral cortex, 1.6 fold increase in the mutant hippocampus and 9 fold increase in the mutant brainstem.

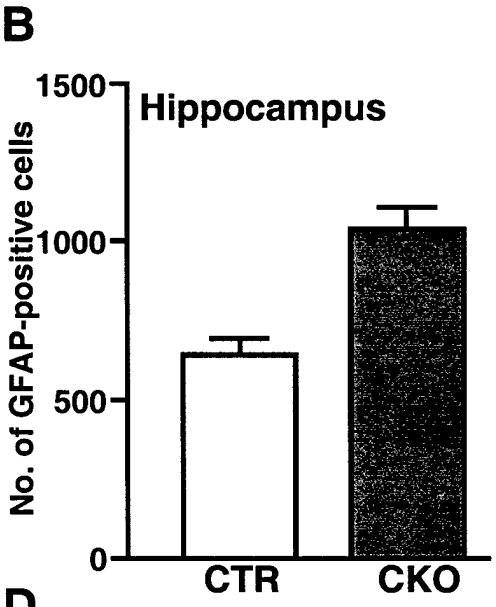
Summary.

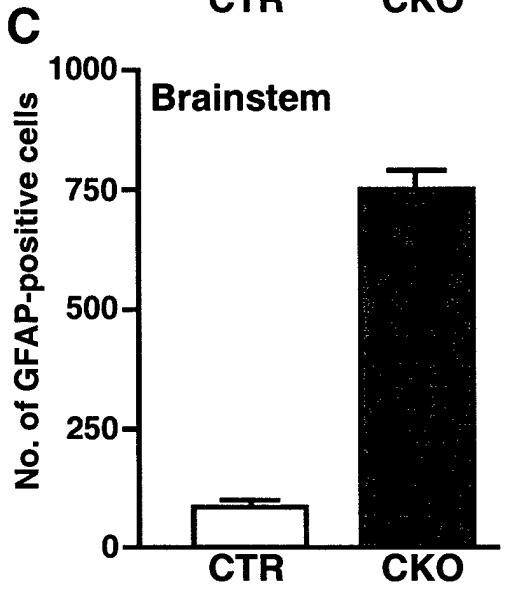
In conclusion, Objective 1 has yielded highly valuable and new information regarding the in vivo function of NF1 as well as a resource that will provide the opportunity to pursue the studies of this interesting gene to address issues of heart development, bone involvement and cancer. Each of the proposed tasks was accomplished

Table 1. Comparison of GFAP staining cells between conditional knock out (CKO) and control (CTR) littermates in: Cortex (A), Hippocampus (B), and Brainstem (C). At the lower left the data is shown as : Mean number of GFAP

Table 1 : Quantitation of GFAP-positive cells







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	CTR	CKO
Cerebral Cortex	774.0 ± 94.4 (4)	3439 ± 67.87 (5)
Hippocampus	645.4 ± 50.15 (5)	1040 ± 63.90 (5)
Brainstem	84.25 ± 14.94 (4)	748.8 ± 39.30 (4)

Objective 2 (Henkemeyer laboratory)

The main goal of this Objective was to generate a conditional null mutation in the mouse *Gap* gene by flanking the two exons encoding amino acids 172 to 267 with Cre recombinase specific *loxP* sequences. At the end of year 1 we reported to have successfully generated the conditional *Gap* targeting vector and, out of 2,883 embryonic stem (ES) cell lines screened, identified 2 lines that exhibited homologous recombination at the *Gap* locus, designated VIII-22 and V-40. This unexpected very low frequency of homologous recombination is in stark contrast to what we previously observed using the pMGAP.G7.7 targeting vector to obtain the null allele where 8 of 108 ES cell lines isolated exhibited homologous recombination (Henkemeyer et al. 1995). We believe that this greatly reduced frequency of homologous recombination may be due to the presence of the repeated *loxP* and *frt* recombination-mediating sequences in the original *Gap* conditional vector, as it is possible their presence may inhibit homologous recombination events at the endogenous *Gap* locus. Nevertheless, we performed Southern blot analysis of purified genomic DNA from the VIII-22 and V-40 ES cell lines and determined that only one of them (V-40) had incorporated both desired *loxP* sequences into the *Gap* gene. In year two, we generated a very large number of chimeric mice with the *Gap* V-40 ES cell line. However, quite unfortunately, none of the chimeric mice made with this cell line transmitted the mutation through the germline. In our extensive experience with ES cell cultures, we find that about 20% of the cell lines used for generating chimeric mice fail to lead to germline transmission. Thus, for most ES cell targeting experiments, we always try to generate chimeras from at least two to three independent cell lines to ensure obtaining a germline transmitting chimera.

Given the key importance of having to obtain germline transmission of a conditional *Gap* mutation, we have in years two and three of this project attempted to obtain additional conditional *Gap* ES cell lines. Unfortunately, over 3,000 more ES cell lines were screened for homologous events following electroporation of the original conditional vector and no other targeted cell lines were recovered. This extremely poor luck at isolating proper targeted ES cells with the original *Gap* conditional vector led us in year three to construct new conditional *Gap* knockout vectors in efforts to obtain one which will provide the necessary homologous recombination events at a higher frequency than 1 out of over 6,000. In these new constructs, we have attempted to use different restriction sites and clone different fragments of the *Gap* gene into the conditional plasmid vector with the aim to attempt to eliminate the regions of the *Gap* genomic DNA that may be interfering with the homologous recombination in the ES cells. Unfortunately, none of these plasmids have provided a single homologous recombination event to date. We do not at the moment have any clear explanation for our failure at obtaining conditional targeting of the *Gap* gene, other than that the presence of the *loxP* and *frt* sequences in the various vectors used, which are essential for making the conditional mutation, must somehow greatly inhibit the homologous recombination at this particular locus. It is important to point out that this failure at knocking out the *Gap* gene is not due to a general inability to do such experiments in the Henkemeyer laboratory, as we have generated a large number of mutations in other genes by homologous recombination in the same ES cells over the course of the past three years and have obtained the germline transmitting chimeric mice. These new mutations are in genes that encode members of the Eph family of receptor tyrosine kinases and their ephrin

ligands (*EphB1*, *EphB2*, *ephrin-B2*, and *ephrin-B3*), in PDZ domain-containing proteins (*Pick-1* and *Lnx*), and in cell cycle regulatory proteins (*Mat-1*, *Ipk*, and *Lkb*). Moreover, four of these successful gene targeting projects utilize the "conditional" approach and include the use of both *loxP* and *frt* sequences. Thus, we have to conclude that, in the case for *Gap*, conditional vectors that contain *loxP* and *frt* sequences somehow greatly reduce the rate of homologous recombination at this particular locus.

Additional studies of GAP and neurofibromin function during the three year period

While we have worked on trying to generate the conditional *Gap* mutation in mice, we have continued to study in detail the phenotypes associated with our original germline null mutation (Henkemeyer et al., 1995). In collaboration with Dr. David Gutmann, we analyzed *Gap/+* heterozygous adult mice to determine whether there is an increase in astrocyte proliferation. While these studies have shown that *NF1/+* heterozygous brains do show some level of increased astrocyte proliferation, the *Gap/+* heterozygotes appeared normal (Gutmann et al., 1999). Thus, astrocyte growth rates appear to be particularly sensitive to the levels of neurofibromin protein, while reduced levels of GAP protein has little or no effect on this process.

During the last three years, we have also studied a novel genetic interaction between *Gap* and a targeted mutation in the *Shp2* gene which encodes a SH2 domain-containing tyrosine phosphatase (Saxton et al. 1997). Both GAP and Shp2 proteins contain SH2 domains and can, therefore, bind with high affinity to other tyrosine phosphorylated proteins, including activated receptor tyrosine kinases. Like *NF1* and *Gap*, *Shp2* is an ancient gene and its Drosophila homolog (called *corkscrew*) has been implicated to function in a number of developmental pathways. Both GAP and Shp2 are implicated in regulating Ras-GTP levels downstream of receptor tyrosine kinases. GAP, a Ras GTPase Activating Protein, functions to accelerate the hydrolysis of GTP-bound Ras to the GDP-bound form, leading to Ras inactivation, while Shp2 is thought to lead to the activation of Ras and MAP kinase signaling. These two proteins are essential for normal development, as mice homozygous for a targeted mutation in either *Gap* or *Shp2* are embryonic lethal by E10. Moreover, Shp2 and GAP appear to be functioning in the same types of cells in the embryo as each of the single mutants exhibits phenotypes affecting posterior elongation, the notochord and vascular development. Moreover, the *Shp2* mutant phenotypes closely resemble those observed following deletion of the Fibroblast growth factor receptor 1 (FGFR1) or one of its ligands, FGF8. We formulated a hypothesis that upon binding of FGF8, the activated FGFR1 transduces a signal into the cell and that these signals are regulated in a negative fashion by GAP and in a positive fashion by Shp2 (**Figure 1**).

To test this hypothesis, we carried out a series of genetic experiments over the past three years to determine whether the elimination of both Shp2 and GAP in the same embryo would suppress the phenotypes associated with the individual single mutants. As Shp2 appears to turn Ras on and GAP appears to turn Ras off, we hypothesized that double mutants might show a more mild phenotype and the embryos would survive to later stages of development. We set up a very large number of crosses with *Gap/+;Shp2/+* compound heterozygous mice and collected well over 500 embryos at various stages of development, focusing at E8.5 to E12.5 days gestation. We needed to collect so many embryos as the double mutants occur at a frequency of 1 in 16. After collection, we utilized two different markers for early embryonic patterning, one for axial mesoderm (*Cordon-bleu*, a marker of the node and notochord) and the other for endothelial cells

(the *Flk-1* receptor tyrosine kinase). As judged by overall morphological appearance and the expression of the markers, the vast majority of *Gap+Shp2* double mutants examined at E9.5 exhibited full suppression of the respective single mutant phenotypes (**Figure 2**). For instance, *Shp2* single mutants always show strong defects in embryo patterning, and the most posterior region of the embryo, the tailbud, is always greatly malformed. Moreover, in our extensive analysis over a six year period and collection of hundreds of *Shp2* single mutants, we have never observed a single mutant to have accomplished the morphological process called turning, where the tailbud turns inward towards the ventral side of the embryo. This defective posterior development associated with the *Shp2/Shp2* single mutant is easily observed by staining embryos for the node/notochord marker, *Cordon-bleu* (**Figure 3**). Quite remarkably, most of the *Gap+Shp2* double mutants showed fairly normal development of the posterior tailbud region (**Figure 3**).

A dosage effect was also evident in that a large fraction of the *Gap/+;Shp2/Shp2* embryos had turned and developed a fairly normal appearing posterior tailbud (**Figure 3**). This indicates that even reducing the amount of GAP protein in the embryo by 50% in the *Gap/+* heterozygous state can greatly reduce the severity associated with the absence of Shp2 function. The genetic suppression was reciprocal in that the *Gap+Shp2* double mutants also appeared to develop further and look healthier than the *Gap* single mutants. In addition to surviving longer in utereo, new previously undescribed phenotypes were observed in the *Gap+Shp2* double mutants, including spina bifida (**Figure 4**). Although this suppression of the early developmental defects observed in the double mutant embryos is quite remarkable, we have not been able to recover such compound mutants surviving to late gestation or birth. Thus, even though *Gap* and *Shp2* appear to work in opposition during the very early stages of embryo growth and patterning, they are still both essential for later stages of embryonic development.

In related genetic experiments, double mutants lacking *Shp2* and the germline null *NF1* mutation did not lead to suppression of the *Shp2* mutant phenotypes. This demonstrates that the genetic interaction of *Gap* and *Shp2* is specific. To complement these genetic studies, biochemical analysis of *Gap* and *Shp2* mutant fibroblasts was preformed and determined that cells lacking GAP show elevated levels of active MAP kinase following exposure to FGF, while cells lacking Shp2 show reduced levels of MAP kinase after stimulation with FGF (**Figure 5**). These results are consistent with our model (**Figure 1**) and indicate that Shp2 and GAP work in concert to control Ras-GTP levels downstream of receptor tyrosine kinases, and that proper regulation of these signals are crucial for early mammalian development. To add one more piece of potentially important data on these studies, we have attempted to visualize where in the embryo this regulation of GTP-bound active Ras signals are most important. To do these experiments, we have used a phospho-specific antibody that specifically recognizes the active (phosphorylated) form of MAP kinase. Wild-type and *Gap/Gap* mutant embryos were collected, fixed and reacted with these antibodies in standard whole-mount immunohistochemistry (IHC) protocols that we have used many time previously with great success (see Henkemeyer et al. 1994). It would be anticipated that the *Gap/Gap* mutant embryos would have higher levels of GTP-bound Ras and, hence, higher levels of the activated phospho-MAP kinase. However, this whole-mount IHC procedure is very sensitive to the particular primary antibody being used and, unfortunately, we have not been able to make these experiments work without a high level of background signal. We are not the only people to try this out on mouse embryos and, indeed, others have failed

equally well, especially when looking at early stages of development (Janet Rossant and Tony Pawson, personal communication). Needless to say, we are just about complete with our data collections and analysis of the genetic interactions of *Gap* and *Shp2*, and are assembling a manuscript to submit these studies for publication in the very near future.

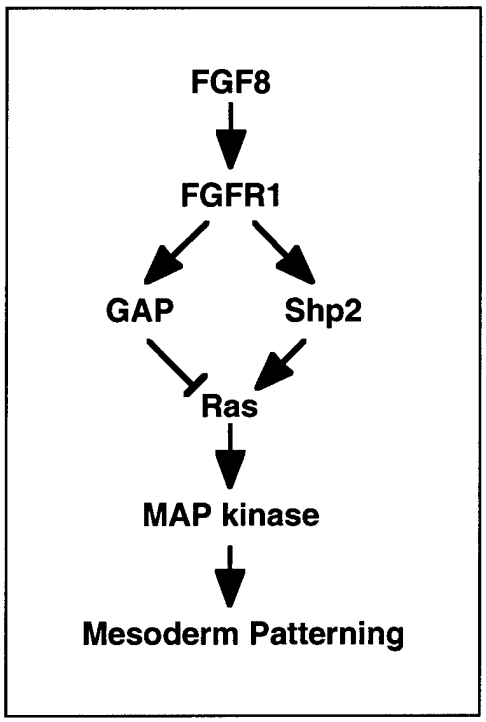


Figure 1. Hypothesized biochemical pathway whereby GAP and Shp2 regulate downstream signaling initiated by FGF8 and FGFR1.

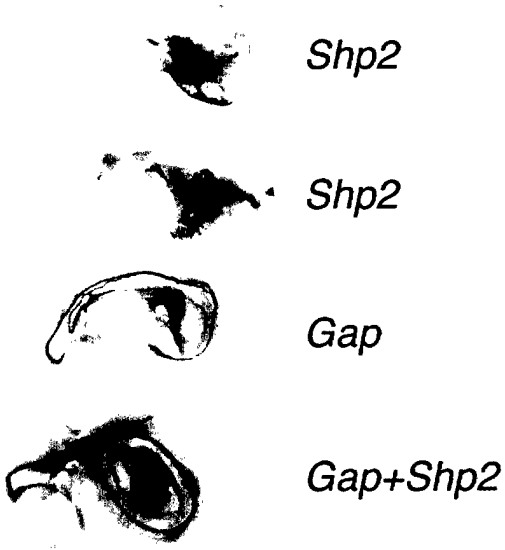
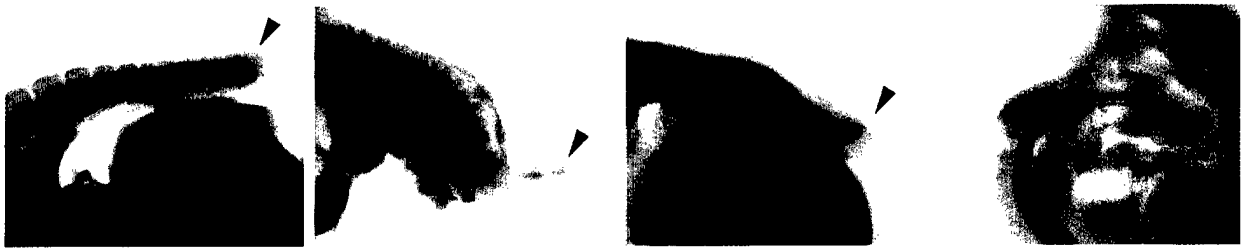
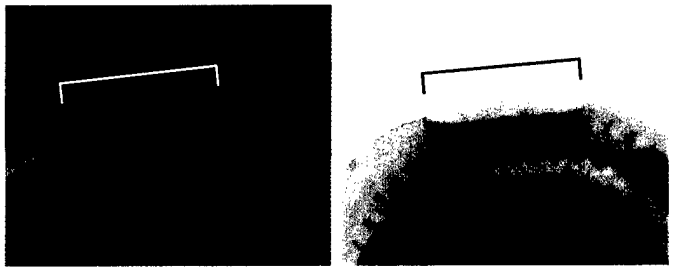


Figure 2. Reciprocal suppression of *Gap* and *Shp2* single mutant phenotypes in *Gap+Shp2* double mutant embryos. Compound heterozygous *Gap/+;Shp2/+* males and females were mated and embryos at E9.5 were collected and stained whole-mount for the *cordon-bleu* beta-gal marker, which labels the notochord and ventral neural tube. Note the complete suppression in the double mutant of the notochord and other morphological defects observed in the individual single mutants.



Gap/+;Shp2/+ *+/+;Shp2/Shp2* *Gap/Gap;Shp2/Shp2* *Gap/Gap;Shp2/Shp2*

Figure 3. Suppression of *Shp2* single mutant phenotypes in the tailbud/posterior-most region of embryos collected at E10.5 days development and stained for the *Cordon-bleu* beta-gal marker, which labels the notochord to the posterior tip of the embryo. Note the near normal development of the tailbud region in the two double mutants shown compared to the *Shp2/ Shp2* single mutant (arrowheads denote the posterior-most region of the embryo).



Gap/Gap;Shp2/+ *Gap/Gap;Shp2/+*

Figure 4. Spinal bifida observed in *Gap/Gap;Shp2/+* embryos collected at E10.5. Shown are two specimens showing a similar open dorsal neural tube (brackets). Such phenotypes have not been observed in *Gap/Gap* single mutants that are wild-type at the *Shp2* locus. In addition to the notochord, the *Cordon-bleu* beta-gal marker also labels a small number of cells in each somite.

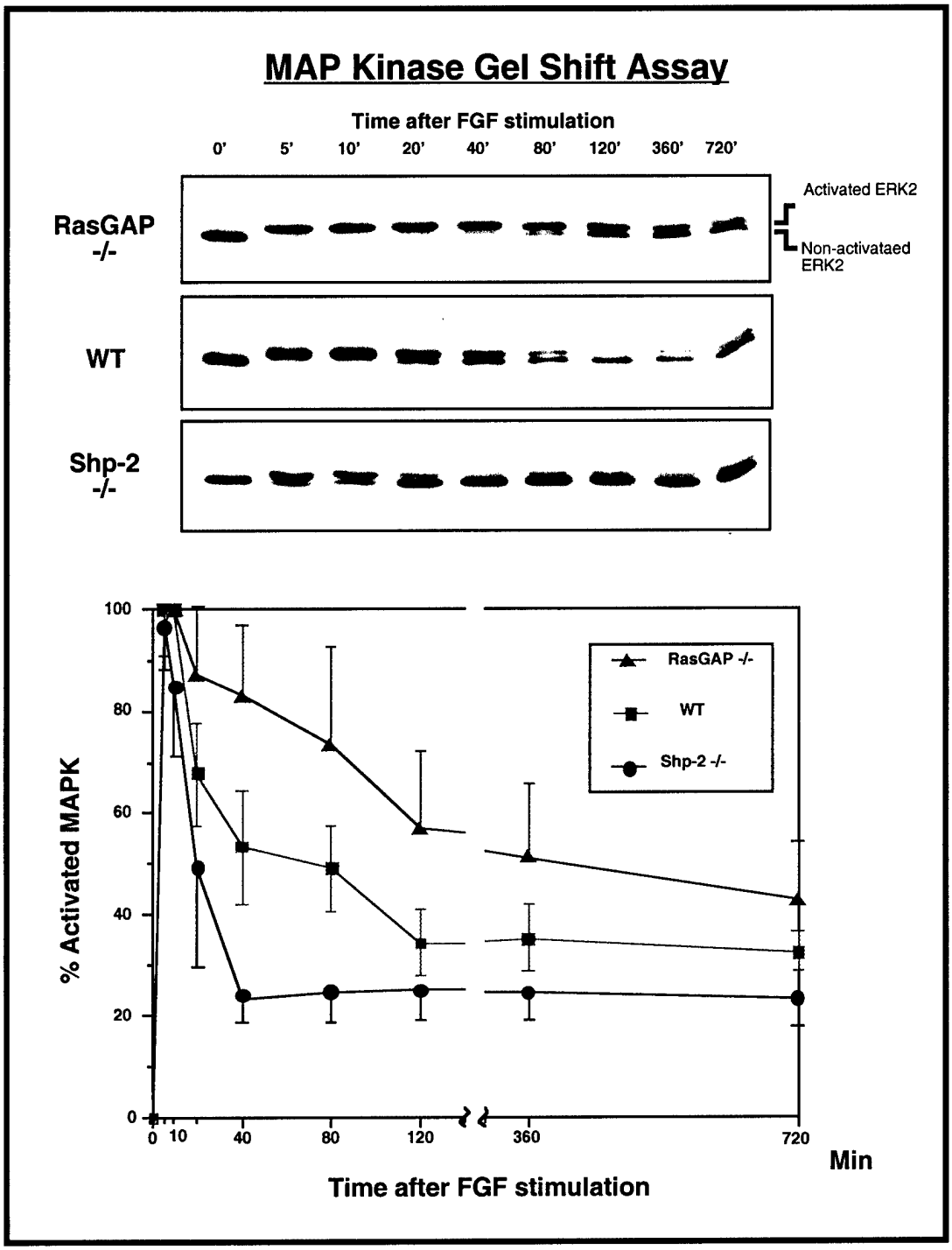


Figure 5. MAP kinase activation in wild-type (green), *Gap* single mutant (red) and *Shp2* single mutant (blue) fibroblast cell lines.

Objective 3. (Henkemeyer) This objective was entirely reliant on the successful generation of the conditional rasGap allele proposed in Objective 2. The proposed experiments within this proposal were directed at the characterization of the mutant mice and the establishment of the degree of redundancy in function between NF1 and rasGap. Consequently the absence of the conditional rasGap allele, precluded the development of this objective.

Objective 4. (Parada)

In this Objective we have proposed to exploit the availability of NF1 and rasGap conditional alleles to examine their contribution in tumorigenesis. This objective has naturally had certain limitations since the NF1 conditional allele only was successfully achieved. However, we have made substantial preliminary progress in exploiting the NF1flox allele in the study of cancer etiology.

NF1 loss in neurons. As outlined in Objective 1, the use of the Synapsin I-cre mouse has permitted the ablation of neurons exclusively. These mice, as described exhibit various phenotypes, including massive astrogliosis. The reason for the presence of reactive astrocytes is uncertain at this point although it is of high interest. A second outcome from this study is that these mice do not develop benign or malignant tumors. These data strongly support the idea that NF1 loss in neurons is not sufficient to engender tumors and also suggest the possibility that NF1 loss in neurons may not be participatory in tumor formation. These issues will be further addressed through combination of NF1 loss in different cell compartments (see below).

NF1 loss in glia. We have employed a GFAP promoter-cre expressing transgenic mouse line to ablate NF1 specifically in GFAP expressing cells. These mice have severe neurological phenotypes that are currently under study. Two clear features arise. First, cerebellar development is perturbed. Second, massive astrogliosis is present in these mice that eventually develop glioblastomas. In consequence, it would appear that selective compartments in the developing CNS are susceptible to loss of NF1. These data are being expanded and will give rise to a unique model for brain tumor formation.

NF1 loss in the neural crest. We obtained a PO promoter- cre transgenic line that has been shown to express cre throughout the neural crest. Interestingly, these mice die with the same timing as do the original NF1 knockout mice. In particular the heart defect in the P0-cre/NF1flox mice appear indistinguishable from the original knock out mouse., In collaboration with Dr. Jon Epstein at University of Pennsylvania medical School, we are expanding these data. The experiments will be elaborated to include a VEGF-cre, and a Tie-2 cre to examine possible roles of endothelial tissues in the heart phenotype.

NF1 loss in Schwann cell precursors. In collaboration with Dr. Patrick Charnay, we have crossed the NF1flox mice to a Krox20 promoter-cre transgenic line. These mjce are anticipated to lose NF1 function in Schwann cell precursors only. The resultant mice exhibit hypermorphic trigeminal and sciatic nerves. We are in the process of examining all peripheral nerve networks to determine the extent of this phenotype. These mice will further be scrutinized for the appearance of tumors in these affected tissues which are the known sites for tumor formation in human patients.

Cooperation of NF1 and p53 in malignant neurofibrosarcoma formation. We have previously shown that compound heterozygous mutant mice at the NF1 and p53 loci develop MPNSTs that resemble human NF1 patient malignancies. To expand on this work, the NF1flozx allele has been place in conjunction with the p53 mutant allele. In this manner we will control the timing and cell type loss of NF1 to determine the spatial and temporal window for NF1 loss in this process.

Summary. In conclusion, the conditional mutation of NF1 has resulted in an unexpectedly fruitful series of projects. As described above, a myriad of hyperplasia and neoplasia models have arisen and are in study. Each of these projects is anticipated to yield important genetic and molecular information about the role of NF1in tumor formation.

Objective 5.

In this objective we proposed to collaborate with Dr. Alcino Silva at UCLA to examine the consequences of neuronal NF1 loss in functioal abd behavioral studies that examine learning and memory. As a first step in this collaboration, we have sent Dr. Silva the Synapsin I-cre mice. These mice have been tested in a preliminary set of behavioral paradigms by Dr. Silva and the preliminary results are very encouraging as the mice exhibit dramatic deficiencies in Morris Water Maze assays as well as deficiencies in hippocampal LTP. These promising data are being reproduced at the same time a second generation of CamII Kinase-cre mice are bred to NF1-flox mice to provide an even more circumscribed model of neuronal loss.

We intend to continue these experiments to their fruition.

Key research accomplishments.

1- Generation of a germline conditional mutation in NF1

2- Demonstration that primary DRG neurons become neurotrophin independent upon cre-mediated recombination.

3. Adult sciatic nerve Schwann cells become spontaneously transformed upon NF1 loss.

4. Loss of NF1 function in neurons provides viable mice. NF1 is not required in neurons for survival.

5. Combination of NFD1 mutation with a p53 mutation leads to neurofibrosarcoma formation.

6. Loss of NF1 in GFAP, P0, Krox20, expressing cells leads to unique phenotypes including tumor formation.

7. Loss of NF1 in neurons appears to cause profound learning and memory deficiencies

8. Successfully generated the conditional *Gap* targeting vector.

9. Used this vector to identified 1 embryonic stem (ES) cell line (V-40) that exhibited correct homologous recombination at the *Gap* locus. Over 6,000 cell lines were screened.

10. Generated a very large number of chimeric mice with the *Gap* V-40 ES cell line. Unfortunately, germline transmission of the conditional mutation was not obtained.

11. Generated new *Gap* conditional targeting vectors, but none provided homologous recombination in ES cells.

12. Studied in detail the phenotypes associated with the original germline null mutation in the *Gap* gene.

13. Discovered and characterized a novel genetic interaction between *Gap* and the gene encoding the SH2 domain-containing tyrosine phosphatase, Shp2.

Reportable Outcomes.

Invited lectures specifically on Neurofibromatosis (Parada)

- The Pathogenesis of NF1 and NF2-Therapeutic Strategies, Banbury Center, Cold Spring Harbor Laboratory, July 14-17.
- Joint Meeting of the Int'l Society for Neurochemistry/American Society for Neurochemistry, Boston, Mass., July 20-26.
- Woods Hole Neurobiology Course, Marine Biological Laboratory, Woods Hole, Mass., July 24.
- Texas Neurofibromatosis Foundation Annual Meeting, Dallas, TX, October 18.
- Society for Neuroscience Annual Meeting, New Orleans, LA, October 24-30.
- Louisiana State Univ. Medical School, New Orleans, November 17.
- 7th Annual European NF Meeting, Paris, France, December 5-6.
- Pasteur Institute, Paris, France, December 11.
- Institut d' Embryologie, Nogent sur Marne, France, December 12.
- Geneva University, Geneva Switzerland, December 15

1998

- Baylor College of Dentistry, Dallas, TX, February 5.
- University of Cincinnati Medical Center, April 16.
- Baylor College of Medicine, Houston, TX, April 28.
- California Institute of Technology, Pasadena, CA, March 18.
- NNFF International Consortium, Aspen, CO, June 7- June 10.
- 1998 FASEB Summer Research Conference, Wilsonville, OR, June 21-26.
- Tuberous Sclerosis Association Conference, Annapolis, MD, July 10-12.
- University of Copenhagen, Denmark, July 30-August 02.
- The Salk Institute, La Jolla, CA, August 15-19.
- MGH Cancer Center Seminar, Boston, MA, September 29-October 01

1999

- Baylor College of Medicine, Department of Human Genetics. Houston, TX. February 22-23.
- Johns Hopkins University. Baltimore, MD.
- Tulane University. New Orleans, LA. April 27-28.
- Texas A&M. College Station, TX. May 4-5.
- Emory University School of Medicine. Atlanta, GA. May 18-20.
- NNFF annual Meeting, Whitehead Institute for Biomedical Research, MIT Cambridge, MA. June 19-22.
- St. Jude Children's Research Hospital. Memphis, TN. Sept. 3-4.
- University of Munich. Munich, Germany. Sept. 19-22.
- 8th Annual European Meeting of the Neurofibromatosis Assoc. Ulm, Germany. Sept. 23-26.

2000

- NNFF Council of Fellows. New York, NY. March 22-24.
- NCI US/Japan Meeting on Tuberous sclerosis. Boston, MA. March 24-26.
- Mayo Clinic. Rochester, MN. April 24-26.
- NNFF Annual Meeting, Aspen CO.
- Carolyn Farb Endowed Lecture on Neurofibromatosis, MD Anderson, August 3.

2001

Coorganizer (with Dr. Zach Hall) of the Annual NNFF meeting Aspen, May 20-23.

Manuscripts.

1) Vogel, K.S., and **Parada, L.F.** 1998. Sympathetic neuron survival and proliferation are prolonged by loss of p53 and neurofibromin. **Mol. Cell. Neuro.**, Vol. 11, 19-28.

3)Klesse L.K., and **Parada, L.F**. 1998. p21 Ras and phosphatidylinositol-3 kinase are required for survival of wildtype and NF1 mutant sensory neurons. **Journal of Neuroscience**, 18 (24), 10420-10428.

4)Klesse L.K., Meyers K.A., Marshall, C.J., and **Parada, L.F.** 1999. Nerve growth factor induces survival and differentiation through two distinct signaling cascades in PC12 cells. **Oncogene**, 18, 2055-2068.

5)Klesse L.K., and **Parada, L.F.** 1999. Trks: Signal transduction and intracellular pathways. Microscopy Research and Technique, 45, 210-216.

6)Vogel, K.S., Klesse, L.J., Velasco-Miguel, S., Meyers, K.A., Rushing, E., and **Parada, LF.** 1999. A mouse tumor model for Neurofibromatosis Type 1. **Science.** Dec. 10; 286(5447), 2176-9.

7)Gonzalez-Zulueta, M., Feldman, A.B., Klesse, L.J., Kalb, R.G., Dillman, J.F., **Parada, L.F.**, Dawson, T.M., and Dawson, V.L. 2000. Nitric oxide activation of $p21^{ras}$/Erk-dependent signaling is required for ischemic preconditioning in neurons. **Proc. Natn. Acad. Sci., U.S.A.** Jan. 4; 97(1), 436-441.

8)Vogel, K.S., El-Afandi, M., and **Parada, L.F**. 2000. Neurofibromin negatively regulates neurotrophin signaling through p21 ras in embryonic sensory neurons. **Mol. Cell. Neuro.**, April; 15 (4), 398-407.

9) **Parada., L.F.** 2000. Neurofibromatosis Type 1. **BBA**, 147, M13-M19.

10) Yuan Zhu, Mario Romero, Pritam Ghosh, Patrick Charnay, Jamey Marth and **Luis F. Parada**. (2000) Ablation of NF1 function in neurons induces abnormal development of cerebral cortex and reactive gliosis in the CNS and PNS. Submitted.

11) Yuan Zhu and **Luis F. Parada** (2000) Do Mice get Human Cancer? Submitted.

12) Klesse, LJ, Meyers, KA, and **Parada, LF.** 2000. Adenoviral mediated interference of the ras-erk signalling pathway blocks NF1/p53 deficient neurofibrosarcoma tumor formation in nude mice. In Preparation.

13) Yuan Zhu, Pritam Ghosh, Elizabeth J. Rushing, Albee Messing and **Luis F. Parada** (2000) Astrocytic Specific Ablation of NF1 Results in Abnormal Neural Development and Tumorigenesis. In preparation.

Key Personnel.

Luis F. Parada
Mark H. Henkemeyer
Chad Cowan
Yuan Zhu
Pritam Gosh
Susana Velasco
Trent Erwin
Laura Klesse
Kristine Vogel
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