Copyright © 2005 by the Genetics Society of America
DOI: 10.1534/genetics.104.038018

A Drosophila Model of Multiple Endocrine Neoplasia Type 2

Renee D. Read,* Paul J. Goodfellow,†,‡ Elaine R. Mardis,‡ Nancy Novak,† Jon R. Armstrong‡ and Ross L. Cagan*,[1]

**Department of Molecular Biology and Pharmacology, †Department of Surgery and ‡Department of Genetics, Washington University School of Medicine, St. Louis, Missouri 63110*

Manuscript received October 31, 2004
Accepted for publication June 7, 2005

ABSTRACT

Dominant mutations in the *Ret* receptor tyrosine kinase lead to the familial cancer syndrome multiple endocrine neoplasia type 2 (MEN2). Mammalian tissue culture studies suggest that *Ret*^{MEN2} mutations significantly alter Ret-signaling properties, but the precise mechanisms by which Ret^{MEN2} promotes tumorigenesis remain poorly understood. To determine the signal transduction pathways required for Ret^{MEN2} activity, we analyzed analogous mutations in the Drosophila *Ret* ortholog *dRet*. Overexpressed dRet^{MEN2} isoforms targeted to the developing retina led to aberrant cell proliferation, inappropriate cell fate specification, and excessive Ras pathway activation. Genetic analysis indicated that dRet^{MEN2} acts through the Ras-ERK, Src, and Jun kinase pathways. A genetic screen for mutations that dominantly suppress or enhance dRet^{MEN2} phenotypes identified new genes that are required for the phenotypic outcomes of dRet^{MEN2} activity. Finally, we identified human orthologs for many of these genes and examined their status in human tumors. Two of these loci showed loss of heterozygosity (LOH) within both sporadic and MEN2-associated pheochromocytomas, suggesting that they may contribute to Ret-dependent oncogenesis.

DURING development, receptor tyrosine kinases (RTKs) integrate extracellular signals to influence cellular processes such as growth and differentiation. Signaling through RTKs requires ligand-induced oligomerization to direct tyrosine autophosphorylation; autophosphorylation both stimulates catalytic activity and creates phospho-tyrosine docking sites for cytoplasmic proteins that activate intracellular signaling pathways. To date, mutations in more than half of all RTKs have been implicated in human cancer (reviewed in Blume-Jensen and Hunter 2001). These mutations commonly function by relieving RTK regulatory constraints, leading to inappropriate kinase activity and hyperactivation of downstream pathways. These events promote oncogenic transformation by driving aberrant cellular growth, proliferation, and survival. Still, tumorigenesis requires mutations in multiple loci: along with dominant mutations in oncogenes such as RTKs, tumorigenesis also requires loss-of-function mutations in tumor suppressors. The relationship between oncogenic tyrosine kinases and tumor suppressors, and the extent to which mutations in each cooperate to direct oncogenic growth, is not well understood.

The *Ret* RTK plays an essential role in both development and oncogenesis. During embryogenesis, *Ret* is required for development of the sympathetic and enteric nervous systems, the neural crest, and the excretory system (Schuchardt *et al.* 1994; Durbec *et al.* 1996; Enomoto *et al.* 2001). The extracellular portion of Ret contains cysteine repeats and a cadherin-like domain. The intracellular portion of Ret contains a tyrosine kinase catalytic domain and multiple tyrosine autophosphorylation sites. Four activating ligands have been identified: GDNF, Neurturin, Persephin, and Artemin all activate Ret through the GPI-linked co-receptors GFRα1-GFRα4 (Jing *et al.* 1996; Kotzbauer *et al.* 1996; Sanchez *et al.* 1996; Buj-Bello *et al.* 1997; Klein *et al.* 1997; Baloh *et al.* 1998; Enokido *et al.* 1998; Enomoto *et al.* 1998; Milbrandt *et al.* 1998). Ligand stimulation of Ret signal transduction activates various signaling pathways and effectors, including Ras/Raf/ERK, PI-3 kinase/AKT, Src, p38-MAPK, Jun kinase (JNK), PLCγ, and ERK5 (van Weering *et al.* 1995; Borrello *et al.* 1996; Chiariello *et al.* 1998; Melillo *et al.* 1999, 2001; Soler *et al.* 1999; Besset *et al.* 2000; Califano *et al.* 2000; Hayashi *et al.* 2000; Encinas *et al.* 2001; Kurokawa *et al.* 2001, 2003). Through these pathways, Ret signaling directs cell differentiation, proliferation, survival, and migration during neural development and renal organogenesis (Schuchardt *et al.* 1994; Heuckeroth *et al.* 1998; Taraviras *et al.* 1999; Enomoto *et al.* 2001; Hashino *et al.* 2001; Natarajan *et al.* 2002; Tang *et al.* 2002).

Dominant, gain-of-function mutations in Ret lead to the familial cancer syndromes multiple endocrine neoplasia type 2A (MEN2A) and type 2B (MEN2B) and

[1]*Corresponding author:* Department of Molecular Biology and Pharmacology, Washington University School of Medicine, 660 S. Euclid Ave., St. Louis, MO 63110. E-mail: cagan@wustl.edu

familial medullary thyroid carcinoma (FMTC). These syndromes are defined by tumors of the endocrine glands arising from neural crest derivatives. MEN2 and FMTC are characterized by the occurrence of medullary thyroid carcinoma (MTC), a malignant tumor derived from thyroid neuroendocrine parafollicular C cells (reviewed in Leboulleux *et al.* 2004). MEN2A and MEN2B patients frequently develop pheochromocytoma, an adrenal neuroendocrine tumor. In addition, MEN2A patients commonly develop parathyroid and skin defects, and MEN2B patients display peripheral nerve and skeletal abnormalities. These syndromes also show variation in age at tumor onset, severity of MTC, and occurrence of other tumors (Easton *et al.* 1989; O'Riordain *et al.* 1995; Ponder 1999). Current treatment is surgery to remove tumors followed by chemotherapy. However, treatment of MTC is difficult since it is prone to metastasis and is often refractory to chemotherapy (Quayle and Moley 2005). Genetic testing in afflicted families has resulted in early identification and prophylactic surgical treatment of individuals with *Ret* mutations (Brandi *et al.* 2001). Yet many cases of MEN2 are attributed to *de novo* mutations, precluding early identification and treatment, and even affected infants can develop MTC (Carlson *et al.* 1994a; Shirahama *et al.* 1998; van Heurn *et al.* 1999).

Mutations in the Ret extracellular region at one of five cysteines occur in almost all MEN2A patients and in approximately two-thirds of FMTC patients (Donis-Keller *et al.* 1993; Mulligan *et al.* 1993b, 1994a,b; Eng *et al.* 1996). MEN2B patients possess mutations that cluster within the tyrosine kinase domain, most commonly a methionine-to-threonine substitution (M918T) and, less frequently, mutations such as A883F or V804M and Y806C/S904C (Carlson *et al.* 1994b; Eng *et al.* 1994; Hofstra *et al.* 1994; Smith *et al.* 1997; Miyauchi *et al.* 1999; Menko *et al.* 2002). Sporadic MTC and papillary thyroid carcinoma also display dominant activating Ret mutations (Grieco *et al.* 1990; Bongarzone *et al.* 1994; Chiefari *et al.* 1998; Scurini *et al.* 1998).

Using primarily tissue culture models, a variety of studies have assessed the biochemical properties of oncogenic Ret to understand the molecular basis for MEN2 diseases. MEN2A forms of Ret (Ret^{MEN2A}) are constitutively dimerized and activated, the result of ligand-independent intermolecular disulfide bonding (Asai *et al.* 1995). In contrast, Ret^{MEN2B} is constitutively activated independently of dimerization and exhibits an altered autophosphorylation pattern (Asai *et al.* 1995; Santoro *et al.* 1995; Liu *et al.* 1996; Iwashita *et al.* 1999). MEN2B mutations likely disrupt normal autoinhibitory function of the activation loop, permitting unfettered kinase activity and substrate binding even in the absence of receptor dimerization (Smith *et al.* 1997; Miller *et al.* 2001). The results are dominant oncogenes that drive transformation due to increased kinase activity and aberrant stimulation of downstream pathways (reviewed in Takahashi 2001). To date, all of the signaling pathways necessary for Ret^{MEN2} transforming activity are also thought to be required for physiological, ligand-stimulated Ret signal transduction (Marshall *et al.* 1997; Ohiwa *et al.* 1997; Chiariello *et al.* 1998; Califano *et al.* 2000; Hayashi *et al.* 2000).

Many outstanding issues remain to be addressed regarding oncogenic Ret. Tissue culture studies have implicated numerous signaling pathways, but little is known about the relevance of these pathways *in vivo*. Moreover, both MEN2 families and MEN2 mouse models display significant phenotypic variation, although the basis for this variation remains largely unexplored (Eng *et al.* 1996; Michiels *et al.* 1997; Acton *et al.* 2000; Kawai *et al.* 2000; Smith-Hicks *et al.* 2000; Cranston and Ponder 2003). Little is known about possible secondary mutations that promote MEN2-associated tumors. For example, only a subset of patients develop pheochromocytoma, implying that second-site mutations in tumor suppressors or susceptibility loci may modulate how different individuals manifest MEN2. With these issues in mind, we established a Drosophila model to address Ret^{MEN2} function in the context of an intact epithelium.

Drosophila has proven a powerful tool for the study of signal transduction pathways in development and disease. In particular, RTKs and their signaling pathways are remarkably well conserved between vertebrates and invertebrates (reviewed in Simon 2000; Voas and Rebay 2004). The Drosophila ortholog of the Ret RTK, dRet, is highly conserved compared to mammalian Ret, as both dRet and mammalian Ret share a 52% protein identity within their SH2 and kinase domains (Sugaya *et al.* 1994; Hahn and Bishop 2001). Interestingly, Ret and dRet are expressed in a number of analogous tissues, including the central and enteric nervous systems and the excretory system (Sugaya *et al.* 1994; Hahn and Bishop 2001), suggesting that dRet can serve as a useful model for understanding the role of Ret in disease as well as development.

To provide insight into Ret^{MEN2}-mediated defects, we created transgenic *dRet* constructs analogous to Ret^{MEN2A} and Ret^{MEN2B}. We chose to target expression of these constructs to the developing fly eye due to its simplicity, its accessibility, and its long history of success as a model system for RTK signal transduction. We demonstrate that these transgenic fly lines exhibit defects associated with human Ret^{MEN2}, including Ras pathway hyperactivation, excess proliferation, and aberrant neuronal differentiation. We performed a genetic screen for genes that dominantly suppressed or enhanced the $dRet^{MEN2}$ phenotypes and identified a large number of genes that participate in dRet signal transduction. Finally, with comparative genomics, we identified human orthologs for some of these genetic modifiers and examined their status in human tumor tissues. Two of these loci were somatically deleted at high rates within both sporadic

and MEN2-associated pheochromocytomas, suggesting that they may contribute to Ret-dependent oncogenesis.

MATERIALS AND METHODS

Cloning *dRet* cDNAs, site-directed mutagenesis, and *GMR-dRet* flies: Full-length *dRet* cDNA clones were isolated from an adult head cDNA library using probes generated to known partial *dRet* cDNA sequences (Sugaya *et al.* 1994). PCR-based site-directed mutagenesis was used to make $dRet^{MEN2B}$ ($dRet^{M955T}$) and $dRet^{MEN2A}$ ($dRet^{C695R}$). For the M955T mutation, codon 955 was changed to ACG from ATG. For the C695R mutation, codon 695 was changed to CGC from TGC. To create *GMR-dRet* transgenes, full-length *dRet* cDNAs were cloned into pGMR. Plasmids were injected into *y w;*Δ *2-3 Ki* flies, and multiple stable insertions were recovered. Isogenic stocks were generated for each independent insertion.

Immunohistochemistry and sectioning: *In situ* hybridization was performed as described (Tautz and Pfeifle 1989). Digoxigenin-labeled DNA probes were made to the *dRet* 5′-end and were detected with alkaline phosphatase conjugated antibodies (Boehringer Mannheim, Indianapolis). For BrdU labeling, live third instar larval imaginal discs were incubated with 8 μg/ml bromodeoxyuridine (BrdU) for 1 hr, fixed in Carnoy's solution, rehydrated, and treated with 2 n HCl in 1× PBS 0.3% Triton X-100. For other immunohistochemical stains, tissue was fixed in 1× PBS 4% paraformaldehyde for 15–20 min. Stains were performed in 1× PBS, 10% serum, 0.3% Triton X-100. 22C10, anti-BrdU, Sevenless, Bar, Boss, and antidiphospho-ERK (Promega, Madison, WI) antibodies were used at 1:100, 1:500, 1:2, 1:25, 1:100, and 1:200, respectively. Staining was detected with Cy3-conjugated secondary antibodies (Jackson ImmunoResearch Labs, West Grove, PA). For adult sections, heads were fixed in 1% glutaraldehyde/2% osmium tetroxide/PBS, dehydrated, and incubated 4 hr in 1:1 propylene oxide: Durcupan ACM resin, overnight in 100% resin, and finally at 65° to harden. Serial sections were stained with 0.5% methylene blue/0.1% toluidine blue. Photographs were taken on a Zeiss Axioplan.

Fly stocks and screens: Fly stocks were obtained from the Bloomington Stock Center unless otherwise noted. $Src64B^{P1}$ was a gift of M. Simon. $Stat92E^{j6C8}$ was a gift of S. Hou. $Ras^{\Delta 40b}$ was a gift of N. Perrimon. *phl* and *Dsor* mutations were gifts of J. Skeath. A deficiency collection of 116 stocks was obtained from Bloomington. A total of 21 deficiency lines containing rough-eye markers, secondary mutations in known *GMR-dRet* modifiers, and/or undetectable balancers were excluded from analysis. A total of 1112 *P*-element insertion lines were from Bloomington and 1412 *P*-element lines were from the Szeged Stock Center. Crosses were performed at 22.5°. F_1 progeny were sorted by visible markers to determine genotype. Eye phenotypes were scored on a dissecting microscope. Modifier penetrance was determined by calculating the percentage of enhanced or suppressed mutation-bearing *GMR-dRet* progeny relative to the total number of mutation-bearing *GMR-dRet* progeny. Crosses were repeated two to six times for lines showing modifier activity to determine if results were consistent. *P*-element lines that consistently showed enhancement or suppression with a penetrance >75% were considered to be $GMR\text{-}dRet^{MEN2B}$ modifiers. For secondary screens, *P*-element flies were mated to tester flies and F_1 progeny were evaluated. Tester strains included *GMR-Gal4* (gift of L. Zipursky), *GMR-p21* (gift of I. Hariharan), *GMR-rpr* (gift of H. Steller), *GMR-hid* (gift of H. Steller), *GMR-sina* (gift of G. Rubin), and $dEGFR^{Elp}$ (gift of N. Baker).

Inverse PCR and sequencing: Genomic DNA sequences flanking the insertion sites of *P*-element modifiers were isolated using an inverse PCR strategy (Spradling *et al.* 1999). Following an initial round of inverse PCR, a second nested PCR was done using products of the first PCR reaction as template. Final PCR products were sequenced using ABI Big Dye terminator cycle sequencing.

Outcrossing, complementation, excision, and additional alleles: *P*-element insertions were outcrossed to *w; h kni e* flies and recombinant males were isolated and mated to *GMR-dRet* females. F_1 progeny were scored for genetic interactions. *P* elements were excised from modifier stocks by crossing to *y w;* Δ*2-3 Ki* and isolating white-eyed progeny. Excision flies were mated to *GMR-dRet* flies and F_1 progeny were scored.

Following sequence analysis, possible additional alleles of *P*-element modifiers were tested. *EP(2)2172*, *EP(3)3003*, Doa^{EP3602}, and $dTor^{EP2353}$ were from Exelixis. $dTor^{\Delta P}$ was a gift of T. Neufeld. *Sin3A* alleles were a gift of D. Pauli. $Ras85^{\Delta c40b}$, $scrb^1$, and $scrib^2$ were a gift of N. Perrimon. $neur^3$, $neur^{11}$, crb^{8F105}, and $Pp1\text{-}87B^1$ were from the Umea Stock Center. $dMi\text{-}2^1$ and $dMi\text{-}2^4$ alleles were a gift of J. Muller. All others were provided by the Bloomington Stock Center. Complementation tests were for lethality only.

To characterize *dMi-2*, $sev\text{-}Ras85D^{v12}$ flies were mated to *dMi-2* mutant flies and F_1 progeny were scored for *dMi-2*-linked enhancement or suppression of $sev\text{-}Ras85D^{v12}$ rough-eye phenotypes.

Loss-of-heterozygosity and sequencing studies on human tumors: Human orthologs for *GMR-dRet* modifiers were identified by sequence comparisons, database searches, and literature. Loss-of-heterozygosity (LOH) analysis was performed using microsatellite repeat markers, essentially as previously described (Peiffer *et al.* 1995). For each locus, the human genomic sequence was searched for long (>15) CA_n repeats. Primers were designed to amplify the CA repeat (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and reactions were optimized. Primers and PCR conditions are available upon request. Briefly, tumor and matched normal blood DNA samples were prepared using conventional phenol-chloroform extractions. Representative portions of the tumor tissues used to prepare DNA were assessed histologically to ensure high neoplastic cellularity. The CA repeat markers were PCR amplified using a forward ^{32}P-labeled primer. The amplified fragments were size separated on polyacrylamide urea gels and visualized with autoradiography. The products for each of the matched tumor-normal pairs were compared and those cases (tumors) for which there was substantial reduction in the relative intensity of one allelic fragment (∼60% or greater reduction) were classified as having LOH.

Sequencing of individual exons from patient and control samples for both TNIK and CHD3 were performed as described previously (Ley *et al.* 2003). Primers chosen for exon 6 of TNIK, which contains a portion of the kinase domain, worked poorly repeatedly and this exon was not sequenced. CHD3 and TNIK sequences were assembled and compared using CONSED and POLYPHRED software (Nickerson *et al.* 1997; Gordon *et al.* 1998). In cases with ambiguous results, sequence tracings for individual tumor and control samples were compared manually.

RESULTS

Characterization of the Drosophila ortholog dRet: On the basis of a published partial *dRet* cDNA, we cloned a full-length *dRet* cDNA that encodes a predicted protein of 1235 amino acids, which has also been

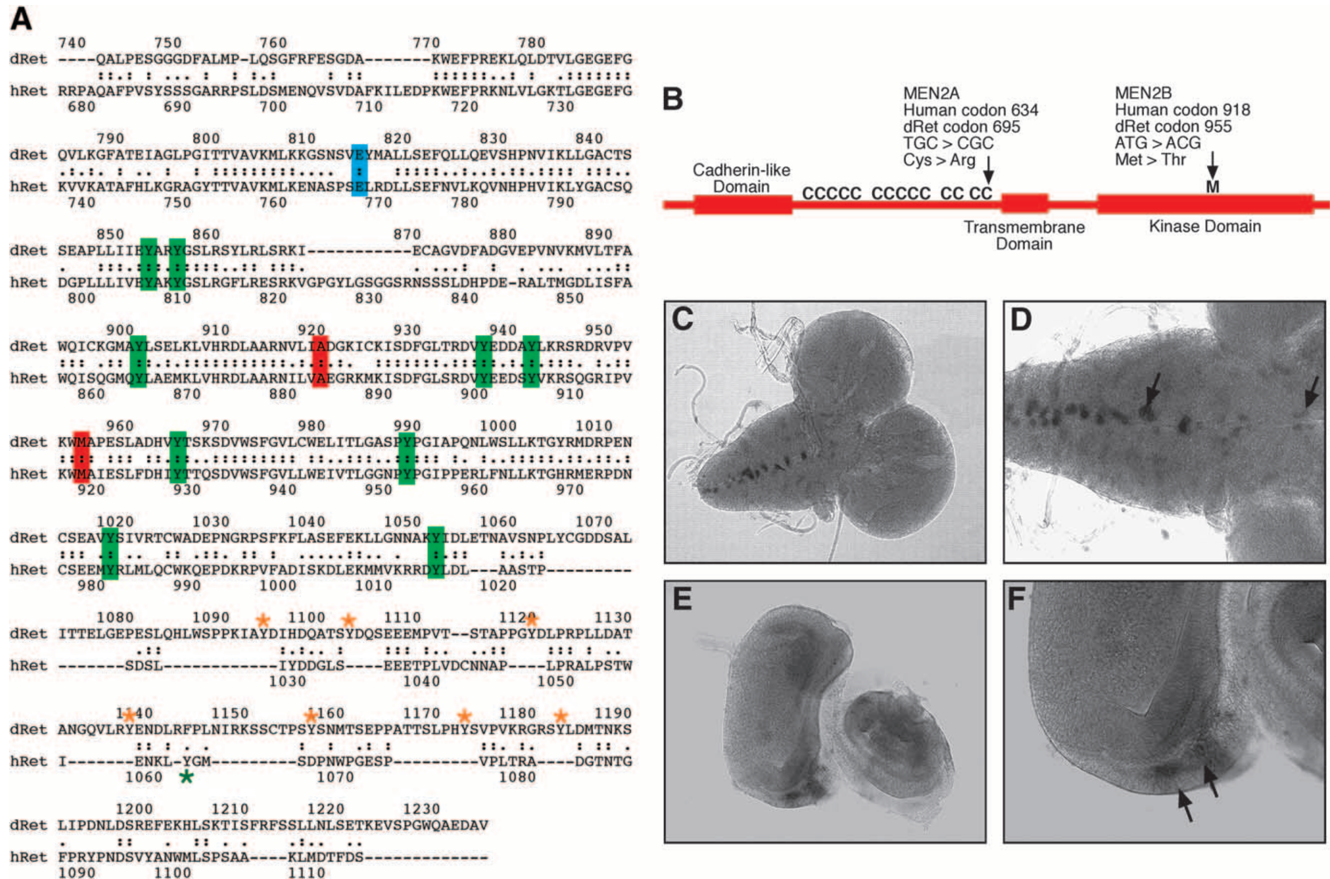

FIGURE 1.—Drosophila *dRet* is highly conserved compared to human Ret. (A) Detailed alignment of dRet (top sequence) and human Ret (bottom sequence) kinase domain and C terminus. Conserved kinase domain residues mutated in MEN2B patients are boxed in red; FMTC is boxed in blue. Conserved tyrosine residues are boxed in green; Y1015 in human Ret is the PLCγ-binding site (BORRELLO *et al.* 1996), human Y900 and Y905 are autoregulatory tyrosines in the activation loop that are required for Ret^{MEN2A} activity, and Y864 and Y952 are required for Ret^{MEN2B} activity (IWASHITA *et al.* 1996, 1999). Y1062 in human Ret, highlighted by a green asterisk, is not strictly conserved in dRet, but the C-terminal tail of dRet is tyrosine rich (orange asterisks) and some of these tyrosines are in motifs that may be Grb2-binding sites. (B) Schematic of dRet protein structure. The cysteine-repeat region contains 14 cysteine residues. Residues mutated to create *dRet*^{MEN2A} and *dRet*^{MEN2B} are noted. (C–F) Larval *dRet* expression. (C and D) *dRet* expression within the larval brain and ventral nerve cord (vnc). Anterior is toward the right. (C) A low-magnification view. (D) A high-magnification view. Note the subset of strongly stained cells in the vnc (arrow) and the lighter-staining cells within the brain (arrow). (E and F) *dRet* expression within the third instar eye-antennal disc. (E) A low-magnification view. The eye field proper is the larger disc to the left; note the diffuse staining that indicates either low-level gene expression or background. (F) A high-magnification view of the *dRet* expressing developing ocelli (arrows), which are adjacent to the eye field.

identified by others (HAHN and BISHOP 2001). The kinase domains of dRet and human Ret (hRet) share 52% identity (Figure 1A). Many hRet autophosphorylation sites are conserved in dRet, including tyrosines required for Ret mitogenic and transforming activities (Figure 1A) (IWASHITA *et al.* 1999). While the extracellular domain of dRet showed only 22% identity with hRet, the dRet and hRet extracellular domains share a common structural organization, which includes a signal sequence, multiple glycosylation sites, a cadherin-like domain, and cysteine repeats (Figure 1B). dRet shows conservation of residues mutated in hRet in MEN2B: human M918 is equivalent to fly M955 and human A883 is equivalent to fly A920. dRet also shows conservation of a residue mutated in FMTC: human E768 is equivalent to fly E815. Within the cysteine repeat region, dRet shows conservation of all cysteine residues reported as mutated in hRet in MEN2A and FMTC: human codons C609, C611, C618, C620, and C634 are equivalent to fly C628, C630, C636, C638, C691, and C695, respectively.

dRet is expressed in the developing central nervous system and peripheral nervous system in embryos (SUGAYA *et al.* 1994; HAHN and BISHOP 2001). In third instar larval tissues, *dRet* expression was observed in a small number of cells in the brain and ventral ganglion (Figure 1, C and D) that morphologically appeared to be neuroendocrine cells (P. TAGHERT, personal communication). *dRet* was also expressed in a restricted pattern in the leg, wing, and antennal imaginal discs (data not shown), but was expressed only weakly or not at all in the eye imaginal disc (Figure 1E). The

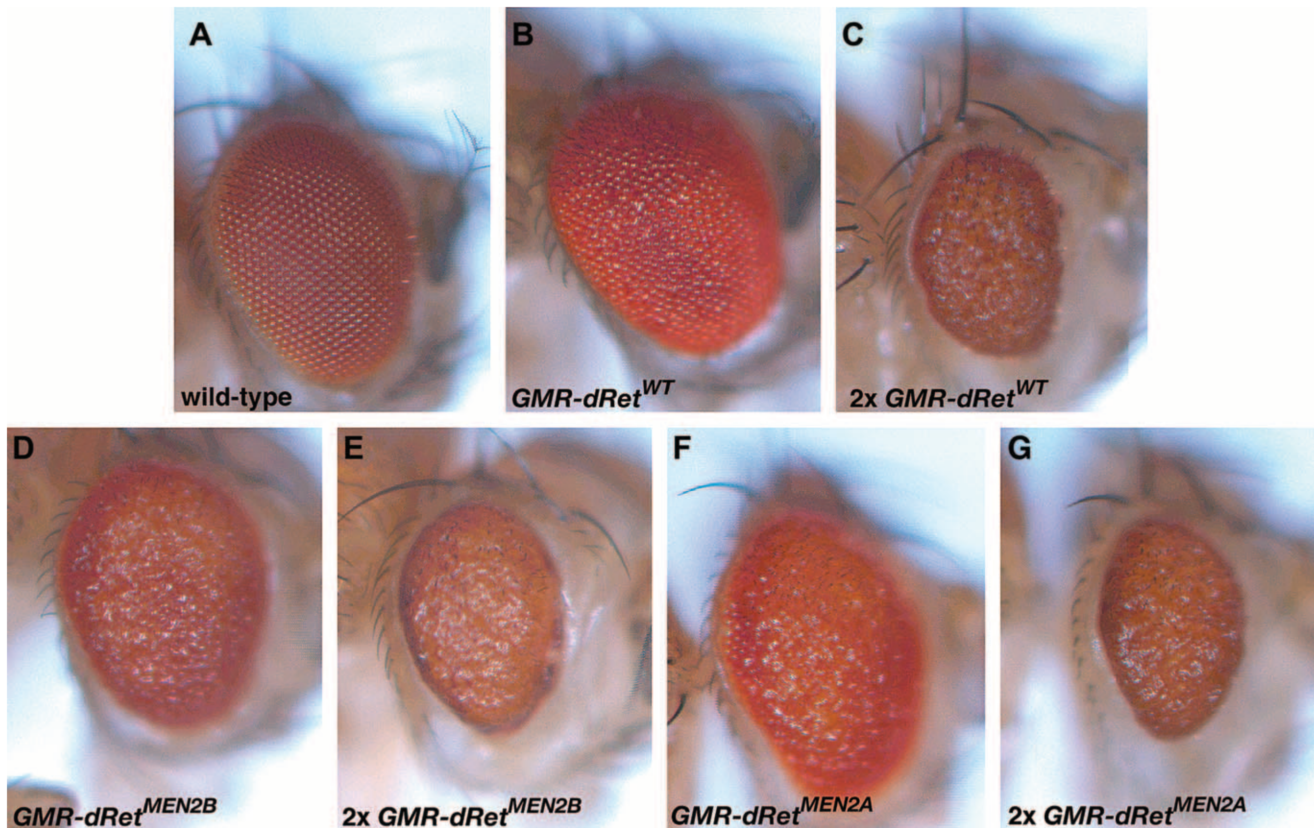

FIGURE 2.—Overexpression of activated dRet causes a dramatic rough eye. $dRet^{WT}$, $dRet^{MEN2B}$ (M955T), and $dRet^{MEN2A}$ (C695R) overexpressed in the retina from the *GMR* promoter. (B, D, and F) Phenotypes conferred by one copy of a *GMR-Ret* transgene, *dRet* isoform indicated. (C, E, and G) Phenotypes conferred by two copies of each *GMR-dRet* transgene.

developing ocelli, which are primitive light-sensing organs, expressed *dRet* as well (Figure 1F).

To date, no ligands or coreceptors for dRet have been described. Our analysis of the Drosophila genome did not uncover any obvious GDNF or GFRα orthologs. One gene, *CG17204*, shows weak similarity to GFRα proteins and contains a putative C-terminal GPI-anchor site, also suggestive of a GFRα ortholog.

dRet overexpression within the developing Drosophila retina: We used a gain-of-function approach to study oncogenic *dRet*. For this purpose, *dRet* was overexpressed in the developing Drosophila retina, a neuroepithelial tissue that shows remarkable evolutionary conservation of RTK signal transduction pathways. The adult retina is composed of ~750 unit eyes known as ommatidia; each ommatidium contains precisely 14 cells. In the late larval stages, eight photoreceptor neurons emerge within each ommatidium in a process that depends on the Drosophila epidermal growth factor receptor (dEGFR) and Sevenless RTKs and the Ras-ERK pathway (Simon *et al.* 1991; Dickson *et al.* 1992; Fortini *et al.* 1992; Biggs *et al.* 1994; Freeman 1996; Karim *et al.* 1996; Spencer *et al.* 1998). Six glial-like cells are then added to complete the 14-cell ommatidium., An interweaving lattice of support cells emerges in the pupa between ommatidia to precisely organize the ommatidial array. This process requires proliferation, cell fate selection, cell morphogenesis, and programmed cell death and is dependent on precisely regulated RTK signaling (*e.g.*, Miller and Cagan 1998; Baker and Yu 2001). Disruption of these precisely choreographed events typically leads to a rough-eye phenotype that is readily scored with a dissecting microscope, making the fly retina an especially useful tool for identifying abnormal gene activity *in vivo*.

To determine if wild-type *dRet* ($dRet^{WT}$) overexpression had an effect in the fly, we fused the $dRet^{WT}$ cDNA to the strong retinal-specific GMR promoter. We created six transgenic fly lines with a stably integrated $GMR\text{-}dRet^{WT}$ construct at various sites throughout the genome. Adults from five of these lines showed either no phenotype or a mildly rough-eye phenotype with a single copy of the transgene (Figure 2B). One $GMR\text{-}dRet^{WT}$ line showed a stronger single-copy phenotype (data not shown), presumably an effect of heightened gene expression due to insertion site. Two copies of $GMR\text{-}dRet^{WT}$ caused a severe phenotype in which the eye became an irregular mass that bulged from the side of the head (Figure 2C), likely due to spontaneous $dRet^{WT}$ activation caused by high expression levels.

To model the effects of the MEN2B mutation, we created $dRet^{MEN2B}$ by engineering a methionine-to-threonine

point mutation at codon 955. *dRet^{MEN2B}* was fused to the GMR promoter and 16 *GMR-dRet^{MEN2B}* stable transgenic lines were created. All exhibited similar phenotypes that were more pronounced than that of *GMR-dRet^{WT}*. One copy of *GMR-dRet^{MEN2B}* caused a dramatic rough eye with disorganized and fused ommatidia (Figure 2D). Two copies of *GMR-dRet^{MEN2B}* yielded a severe phenotype in which the eye became an irregular mass (Figure 2E). Interestingly, similar phenotypes have been observed with retinal specific overexpression of Ras85D^{V12}, an activated version of Ras85D, and Keren, a dEGFR ligand that activates the dEGFR pathway (Bishop and Corces 1988; Karim *et al.* 1996; Halfar *et al.* 2001; Reich and Shilo 2002).

Finally, to investigate MEN2A, we created transgenic flies that overexpress a MEN2A form of *dRet* (*dRet^{MEN2A}*). Site-directed mutagenesis was used to create a cysteine-to-arginine mutation at codon 695; this position is orthologous to cysteine 634 in human Ret, the most commonly mutated residue in MEN2A patients. Twelve stable lines of *GMR-dRet^{MEN2A}* flies were created. All showed a phenotype nearly identical to that of *GMR-dRet^{MEN2B}* and clearly stronger than that of *GMR-dRet^{WT}* (Figure 2F), suggesting that both MEN2 forms of *dRet* are hypermorphs. Two copies of *GMR-dRet^{MEN2A}* conferred a phenotype indistinguishable from the effects of two copies of *GMR-dRet^{MEN2B}* (Figure 2G). Two copies of *GMR-dRet^{WT}* and of either *GMR-dRet^{MEN2}* isoform produced similar phenotypes, suggesting that the MEN2 isoforms are not neomorphic, although we cannot rule out subtle effects.

Phenotypic analysis of dRet overexpression lines: Given the similarities among *GMR-dRet^{MEN2B}*, *GMR-dRet^{MEN2A}*, and *GMR-dRet^{WT}* adult phenotypes (and their genetic similarities; see below), we selected one transgene to examine in greater detail. Cell fate differentiation and GMR expression begin in the larval eye disc behind the anterior sweep of the morphogenetic furrow. As the first neurons emerge behind the furrow, undifferentiated cells undergo a coordinated wave of mitosis referred to as the "second wave." In *GMR-dRet^{MEN2B}* retinae, excess S-phase cells were observed within and ahead of the second wave, where the *GMR* promoter is active (Figure 3, A and B). Cobalt sulfide preparations also indicated excess mitosis in *GMR-dRet^{MEN2B}* larval retinas compared to wild-type controls (data not shown). However, *GMR-dRet^{MEN2B}* retinas did not appear greatly enlarged relative to wild-type controls; this may be because *GMR-dRet^{MEN2B}* larval retinas also showed increased apoptosis, as seen with acridine orange staining (data not shown).

Differentiation of the first neuronal cell type, R8, was unaffected by *GMR-dRet^{MEN2B}* (Figure 3, C and D); this was expected, as R8s begin differentiation prior to gene expression from the *GMR* promoter. Other types of photoreceptor neurons exhibited abnormal specification and patterning following the onset of *dRet^{MEN2B}* expression, as assessed by staining with antibodies to the photoreceptor-type-specific proteins Bar and Sev (Figure 3, E–H). Furthermore, the antibody 22C10, which highlights all photoreceptor neurons in the retina, demonstrated that cells between ommatidia were ectopically and inappropriately differentiating into neurons in response to *GMR-dRet^{MEN2B}* (Figure 3, I and J). Ectopic neuronal differentiation is a defect previously observed with aberrant Ras pathway activation in the fly eye (Van Vactor *et al.* 1991; Dickson *et al.* 1992; Fortini *et al.* 1992; Lai and Rubin 1992). Consistent with hyperactivation of the Ras pathway, *GMR-dRet^{MEN2B}* larval retinae displayed excessive levels of active diphospho-ERK (Figure 3, K and L). Mammalian studies indicate that Ret^{MEN2B} aberrantly activates the Ras pathway to trigger ERK phosphorylation and activation (Ohiwa *et al.* 1997; Califano *et al.* 2000; Salvatore *et al.* 2001), and MEN2 mutations can lead to excessive neuronal proliferation and differentiation (Wolfe *et al.* 1973; Matias-Guiu *et al.* 1995).

Later retinal development in *GMR-dRet^{MEN2B}* flies was severely affected, indicating that the retinal phenotype became progressively worse over time. For example, an antibody to the junctional marker Armadillo, which outlines the apical profiles of cells, showed that support cells (*e.g.*, pigment cells) in *GMR-dRet^{MEN2B}* pupal eyes failed to attain a normal mature morphology (Figure 4, A–C). *GMR-dRet^{MEN2A}* pupal eye tissue showed indistinguishable defects (data not shown). Similar pupal eye phenotypes have been reported for Ras85D^{V12} overexpression (Sawamoto *et al.* 1998). Histological sections of adult *GMR-dRet^{MEN2B}* retinae showed variable numbers of photoreceptors, poorly spaced ommatidia, and large vacuolated spaces (Figure 4D). Later-stage *GMR-dRet^{MEN2A}* and *GMR-dRet^{MEN2B}* adult eyes typically exhibited degeneration (data not shown), indicative of a further progression of *GMR-dRet^{MEN2}*-mediated defects.

The Ras, Src, and JNK pathway members genetically interact with *GMR-dRet^{MEN2}* isoforms: Our phenotypic analysis of *GMR-dRet^{MEN2B}* eyes suggested they have several cellular defects that have been associated with mammalian Ret^{MEN2}, including Ras pathway activation and excess proliferation. With these data in hand, we concluded that the *GMR-dRet^{MEN2B}* and *GMR-dRet^{MEN2A}* flies presented a novel and useful opportunity to examine Ret^{MEN2} function with the powerful genetic tools available in Drosophila. Importantly, a twofold increase in the dosage of any *GMR-Ret* construct increased the severity of the retinal phenotype, suggesting that *GMR-Ret* phenotypes would be sensitive to altered dosage of endogenous genes. To validate our Drosophila model of Ret^{MEN2} signaling and to justify broader, unbiased screens, we examined genetic interactions between *GMR-dRet^{MEN2B}*, *GMR-dRet^{MEN2A}*, and/or *GMR-dRet^{WT}* and mutations in orthologs of genes previously implicated in oncogenic mammalian Ret function (Table 1).

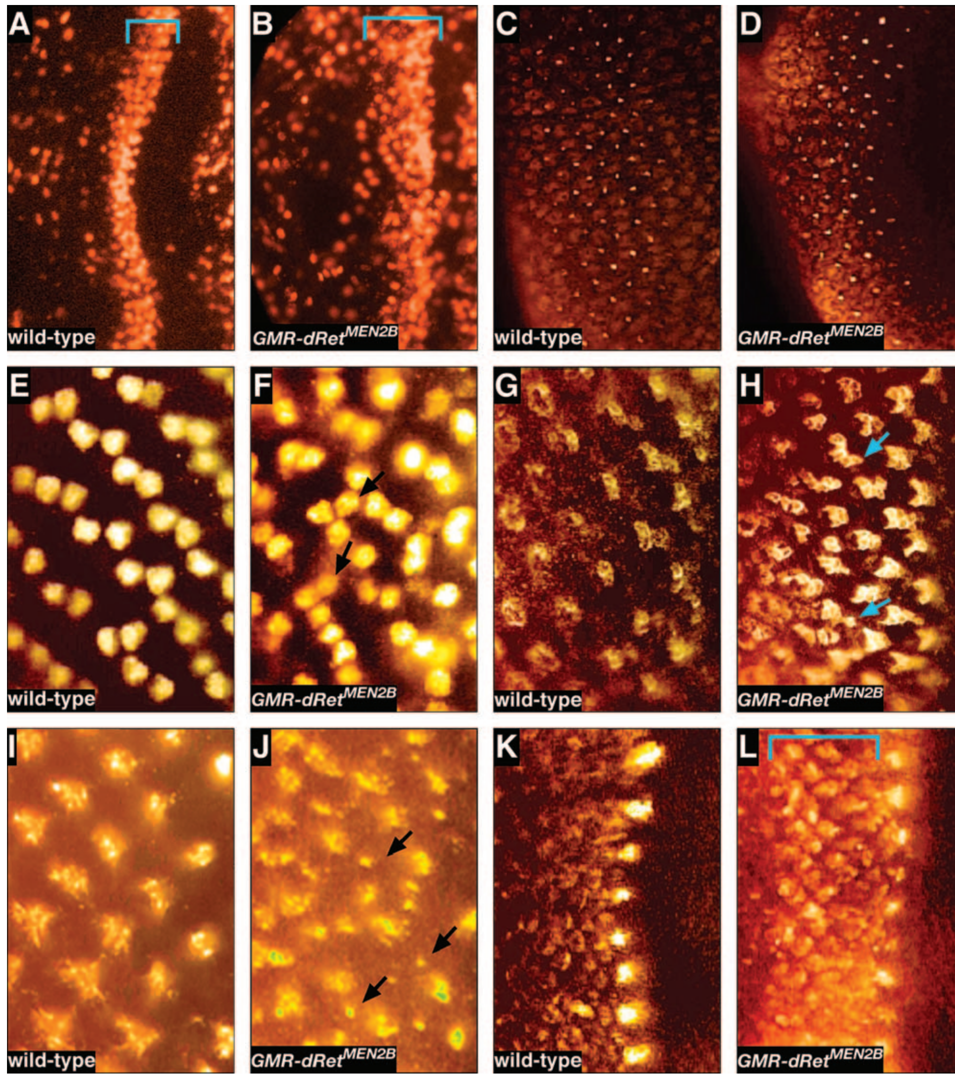

Figure 3.—*GMR-dRet*^{MEN2B} directs excess proliferation, patterning defects, excess neuronal differentiation, and ectopic ERK activation. Anterior is toward the right. (A and B) The larval eye field differentiates in a posterior-to-anterior wave, and the brackets denote the second wave of mitoses; S-phase nuclei are visualized with BrdU (orange); note the ectopic S-phase cells in *GMR-dRet*^{MEN2B} tissue. (C and D) Eye discs from *GMR-dRet*^{MEN2B} flies (two copies) display a normal pattern of Boss staining (orange), indicating that early photoreceptor development (R8) is normal. Staining for Atonal (another marker for R8) in *GMR-dRet*^{MEN2B} tissue is also normal (data not shown). These stains show that early development proceeds normally before *GMR*-driven *dRet* expression begins and that R8 retains its normal identity. (E and F) Eye discs from *GMR-dRet*^{MEN2B} flies (two copies) contain disorganized Bar-expressing cells (arrows in F), suggesting aberrant recruitment, specification, or patterning of later-developing photoreceptor cells R1 and R6. (G and H) Eye tissue from *GMR-dRet*^{MEN2B} flies (two copies) stained for Sev protein (orange) shows ectopic and aberrant clusters of Sev-expressing cells (arrows in H). Note that older, more posterior clusters of Sev-expressing cells are improperly spaced. (I and J) Photoreceptor neurons are visualized with 22C10, a neuronal-specific antibody. *GMR-dRet*^{MEN2B} (two copies) eye tissue contains ectopic neurons (arrows in J) between abnormally spaced ommatidia containing variable numbers of neurons. (K and L) An antibody specific for activated, di-phospho-ERK protein indicated that *GMR-dRet*^{MEN2B} (two copies) eye tissue contains high levels of activated ERK in irregular patches (bracket in L) posterior to the morphogenetic furrow after the onset of *GMR*-induced expression. Early di-phospho-ERK staining (anterior to bracket in L) is normal in *GMR-dRet*^{MEN2B} discs prior to the onset of *GMR*-induced expression.

Ras pathway: Ligand-mediated or oncogenic activation of Ret leads to binding of adaptor proteins, recruitment of the Grb2/SOS complex, activation of Ras, and induction of ERK activity (van Weering *et al.* 1995; Ohiwa *et al.* 1997; Chiariello *et al.* 1998; Hayashi *et al.* 2000; Kurokawa *et al.* 2001; Melillo *et al.* 2001). The Ras pathway is remarkably well conserved between mammals and Drosophila. In a series of tests, we found that *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2B}, and *GMR-dRet*^{MEN2A} all genetically interacted with Ras pathway components (Table 1). Mutations in *drk* (Grb2), *Sos*, *Ras85D*, and *ksr* all alleviated ("suppressed") *GMR-dRet*-mediated phenotypes. Conversely, *GMR-dRet*^{MEN2B} and *GMR-dRet*^{MEN2A} phenotypes were made more severe ("enhanced") by mutations in *Gap1* (RasGAP), a negative regulator of Ras.

GMR-dRet^{MEN2B} failed to interact with more downstream components of the Ras pathway, such as *phl* (Raf). Similar results have been observed in modifier screens with the Sevenless RTK, illustrating that retinal phenotypes caused by activated RTKs are sensitive primarily to the gene dosage of upstream members of the Ras pathway. Mutations in the locus encoding the dEGFR ligand Spitz also suppressed *GMR-dRet* phenotypes, suggesting that reduction in parallel RTK signaling can inhibit the effects of dRet^{MEN2}. Together, these data indicate that *dRet* overexpression led to activation of the Ras pathway, consistent with our immunohistochemical observations. Furthermore, these data demonstrate that *GMR-dRet* flies can identify biochemical pathways linked to mammalian Ret^{MEN2}.

Src and Jun kinase pathways: GDNF-stimulated mammalian Ret, Ret^{MEN2A}, and Ret^{MEN2B} promote Src kinase activation in tissue culture (Melillo *et al.* 1999; Encinas *et al.* 2001). Src activity, in turn, is required for

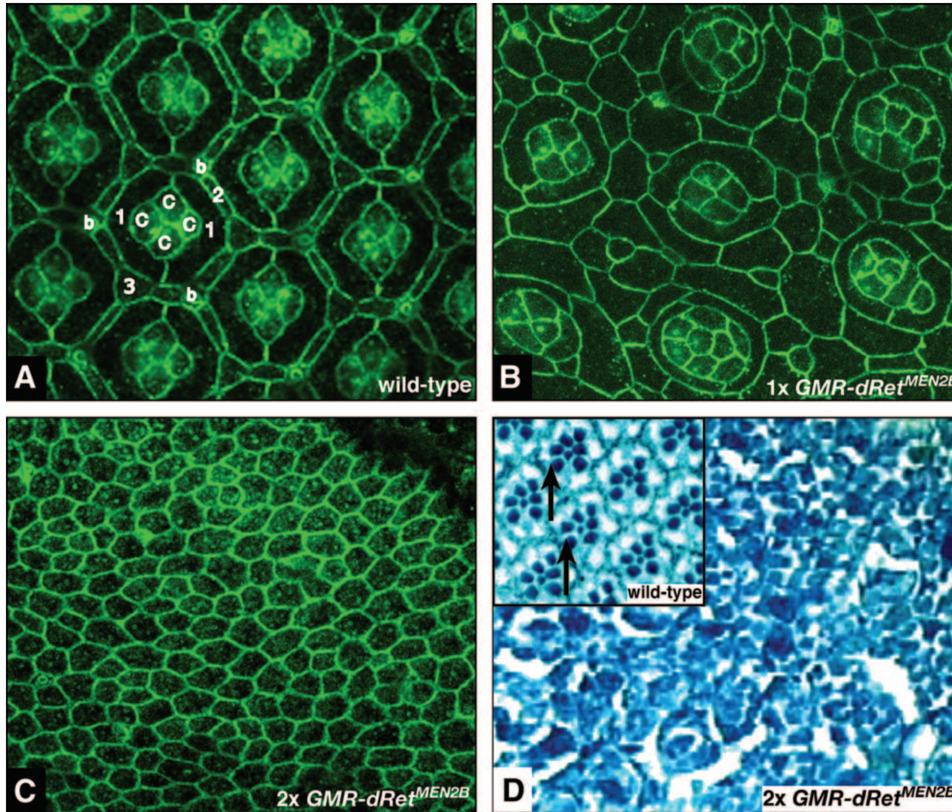

FIGURE 4.—Abnormal patterning and differentiation of ommatidia and interommatidial lattice cells in more mature *GMR-dRet*^{MEN2B} retina. (A and B) Forty-two-hour pupal eye imaginal discs; apical profiles of cells are visualized with an antibody specific for the junctional protein Armadillo. (A) A wild-type pupal eye showing approximately a dozen ommatidia. Cells within and surrounding one ommatidium are labeled to indicate cone cells (c) and primary (1), secondary (2), and tertiary (3) pigment cells, and bristles (b) define an interommatidial lattice. (B) A single copy of *GMR-dRet*^{MEN2B} resulted in a milder phenotype that included abnormal numbers of cone cells and a poorly patterned interommatidial lattice. (C) *GMR-dRet*^{MEN2B} (two copies) eyes show a marked lack of ommatidial organization including an absence of clearly definable cells. The rounded cuboidal shape of these cells is typically seen in undifferentiated lattice cells early in normal pupal retinal development. (D) Plastic sections from adult eye tissue. Wild type (inset) has a normal complement of seven photoreceptor neurons within each ommatidium; they are most easily seen by their rhabdomeres, which appear as solid blue circles in the section. Note that each rhabdomere array forms a stereotyped trapezoid that "points" upward (arrows in inset). *GMR-dRet*^{MEN2B} (two copies) sections contain abnormally assembled and patterned ommatidia and large vacuolated spaces.

Ret-mediated cell survival, proliferation, and activation of ERK and AKT kinases, which themselves function in oncogenic Ret signaling (Chiariello *et al.* 1998; Melillo *et al.* 1999; Murakami *et al.* 1999b; Segouffin-Cariou and Billaud 2000; Encinas *et al.* 2001). Mutations in both Drosophila Src orthologs, *Src42A* and *Src64B* (Takahashi *et al.* 1996; Dodson *et al.* 1998), suppressed *GMR-dRet*^{MEN2A} and *GMR-dRet*^{MEN2B} (Table 1), indicating that ectopic dRet requires Src activity for its function.

GDNF-stimulated Ret, Ret^{MEN2A}, and Ret^{MEN2B} are also known to promote JNK activation and c-jun phosphorylation in numerous cell lines (Marshall *et al.* 1997; Chiariello *et al.* 1998; Murakami *et al.* 1999a; Hayashi *et al.* 2000). This activity requires the Nck adaptor protein (Murakami *et al.* 1999a). The Drosophila genome contains a single JNK ortholog, *basket* (*bsk*), and a single c-jun ortholog, *Jra.* Both *bsk* and *Jra* mutations suppressed *GMR-dRet*^{MEN2A} and *GMR-dRet*^{MEN2B} (Table 1), indicating that dRet also requires JNK pathway activity. The *dreadlocks* (*dock*) locus encodes a Drosophila Nck ortholog (Garrity *et al.* 1996); *dock* mutations failed to modify *GMR-dRet*^{MEN2B} (Table 1). JNK signaling can also be activated by *dishevelled* (*dsh*) (Boutros *et al.* 1998), but mutations in *dsh* failed to modify *GMR-dRet.*

***dRet* modifier screens:** Our data establish the *GMR-dRet* flies as a functional model for Ret signal transduction. Differences between the Ret^{MEN2} isoforms have been proposed on the basis of previous mammalian work (Songyang *et al.* 1995). To explore this issue, we performed comparative genetic screens to identify factors common to all *dRet* isoforms and factors that distinguish one from another. We were especially interested in those genes that modified both *GMR-dRet*^{MEN2} isoforms, as these would help identify (i) candidate factors that mediate *Ret*^{MEN2} transformation, (ii) potential "susceptibility loci" and/or tumor suppressors that promote *Ret*^{MEN2}-dependent tumors when mutant, and, finally, (iii) attractive therapeutic targets for treatment of MEN2 tumors.

To this end, we used a genetic modifier screen to identify second-site loci that enhanced or suppressed the *GMR-dRet* phenotypes. Modifiers represent genes that act as positive or negative regulators of dRet. We screened for dominant genetic modifiers. That is, we used heterozygotes: by altering only one genomic copy of each candidate gene, we were able to test loci that are lethal when homozygous mutant. This approach allowed us to identify truly novel functional dRet^{MEN2} partners, providing an important *in situ* complement to biochemical studies

TABLE 1

***GMR-dRet**^{MEN2}* **genetically interacts the Ras, Src, and JNK pathways**

Gene	Gene function	Allele	*GMR-dRet*^{WT}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{MEN2B}
spitz	dEGF receptor ligand	*spitz*^{s3547}		WS(65)	WS(86)
		spitz^{01068}			WS(46)
		spitz^{1}		WS(40)	WS(56)
drk	Ortholog of Grb2 protein	*drk*^{k02401}	N	N	SS(95)
		drk^{k13809}	WS(23)	N	WS(100)
		drk^{10626}	MS(100)	WS(24)	SS(100)
Sos	RasGTP-exchange factor	*Sos*$^{34Ea-6}$			SS(100)
Gap1	RasGTPase activating factor	*Gap1*^{B2}	ME(86)	SE(100)	SE(97)
Ras85D	Ras ortholog	*Ras85D*^{06677}		MS(100)	WS(76)
		Ras85D$^{\Delta c40b}$			WS(67)
ksr	Kinase suppressor of Ras	*ksr*$^{S-627}$	N	MS(100)	
		ksr^{j5E2}	WS(18)	WS(84)	WS(67)
phl	Raf kinase	*phl*$^{11.29}$			N
		phl^{PB26}			N
Dsor	MAP kinase kinase	*Dsor*^{1}			N
Src64B	Src kinase ortholog	*Src64B*^{P1}		WS(86)	WS(65)
Src42A	Src kinase ortholog	*Src42A*^{k10108}		WS(50)	WS(74)
bsk	c-jun kinase (JNK)	*bsk*^{1}		WS(47)	WS(44)
		bsk^{2}		WS(43)	WS(86)
Jra	c-jun transcription factor	*Jra*^{IA109}	WS(46)	WS(56)	WS(61)
dock	Ortholog of Nck adaptor	*dock*^{k13421}			N
		dock^{04723}			N
dsh	Wnt, JNK signaling	*dsh*^{3}		N	N
		dsh^{6}	N	N	N

Genetic interactions are indicated according to strength of the phenotype: W, weak; M, moderate; and S, strong; and the type of interaction: S, suppressor; E, enhancer; N, no interaction. WE, for example, indicates a weak enhancer. Parentheses indicate penetrance of the interaction as a percentage.

that have relied on testing candidate factors to identify Ret^{MEN2} effectors.

The screen design is diagrammed in Figure 5. Our first screen focused on genomic deficiencies, each of which typically removes dozens of genes. *GMR-dRet*^{MEN2B}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{WT} flies were crossed to flies heterozygous for deficiency mutations; each deficiency was maintained over a wild-type "balancer" chromosome. We compared the following F_1 progeny: *GMR-dRet*; $+/+^{balancer}$ *vs. GMR-dRet*; *Deficiency*/+; *GMR-dRet*; *Deficiency*/+ *vs. GMR-dRet*; and +/+ when appropriate to control for balancer effects. Phenotypic differences between the two progeny types indicated that the mutations tested were genetic modifiers of *GMR-dRet*.

Comparative deficiency screen: We tested all three *GMR-dRet* isoforms using a set of 95 deficiencies that remove ~65% of the genome. Twenty-eight deficiencies that define at least 20 separate genomic regions genetically modified all three *dRet* isoforms (Tables 2 and 3). Candidate modifier genes for these regions were then identified through database searches and directly tested for genetic modification of the *GMR-dRet* isoforms (Tables 2 and 3). In some cases deficiencies overlapped, making identification of modifier regions more precise. We concentrated on candidate genes that regulate signal transduction, growth, proliferation, or differentiation within the eye. One caveat is that, for deficiencies for which we could not identify matching modifier loci or overlapping modifier deficiencies, we cannot be confident that the modifier activity was caused by the deficiency or by another mutation elsewhere on the chromosome. Nevertheless, we identified 11 modifier loci with this approach, providing a crucial proof of principal for a broader, unbiased screen of single gene mutations.

Deficiency screens: suppressors of *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B}: Nine genomic regions that contain *GMR-dRet* suppressors were identified. Several of these regions contain known regulators of Ras signaling. For example, *Df(3R)by10*, a suppressor of both *GMR-dRet*^{MEN2} isoforms, deletes 85D8;85F1 (Table 2), a region that contains the Ras ortholog *Ras85D*. The suppressing deficiencies *Df(2L)net-PMF* (21A1;21B8) and *Df(2L)al* (21B8;21D1) contain the genes *kismet* and *ebi*, respectively (Table 2). Both *kismet* and *ebi* are involved in chromatin remodeling and are positive regulators of dEGFR and Ras signaling in the retina (Therrien *et al.* 2000; Tsuda *et al.* 2002). Mutations in either locus suppressed *GMR-dRet* phenotypes.

Two interacting deficiencies are of special note. *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B} were all suppressed by *Df(2L)TW161*, which removes a region that includes the endogenous *dRet* locus (Table 2). Reduced endogenous *dRet* expression could suppress *GMR-dRet*

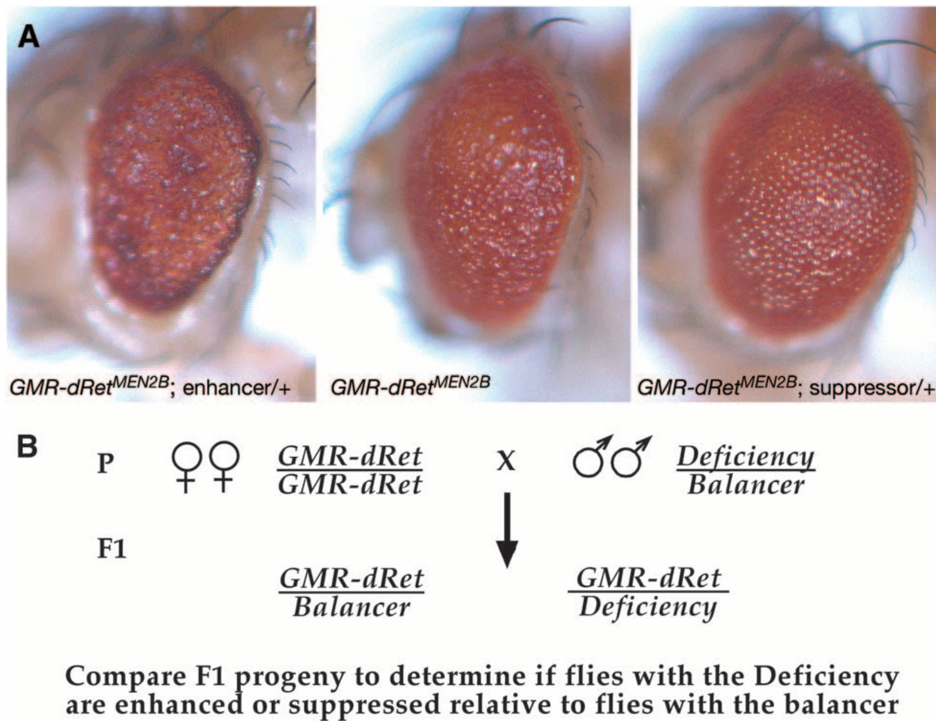

Figure 5.—Enhancer-suppressor screen for modifiers of *GMR-dRet*^{MEN2}. (A) Examples of *GMR-dRet*^{MEN2B} modifier phenotypes, which were scored using a dissecting microscope. Enhanced flies had a worsened phenotype and suppressed flies had a milder phenotype. (B) Diagram of parental (P) genetic crosses and F_1 progeny genotypes. *GMR-dRet*^{MEN2B}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{WT} flies were crossed to flies heterozygous for loss-of-function deficiency mutations; each mutation was maintained over a wild-type balancer chromosome (balancer chromosomes contain breakpoints that prevent meiotic recombination and carry visible markers, but are otherwise genetically wild type and are denoted as such here). The phenotypes of different classes of progeny were compared and the penetrance was calculated for any observed genetic interactions.

phenotypes by reducing overall *dRet* levels and would indicate that dRet is active in the retina. However, no *dRet* mutations are currently available to test this possibility. Also, *Df(3R)DG2* and *Df(3R)Cha7* define a region (91B2;91F5) that includes *glass*, the transcriptional activator that drives gene expression from the *GMR* promoter. As expected, loss-of-function *glass* mutations were confirmed as *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B} suppressors, presumably due to reduction in *GMR* activity.

TABLE 2

Deficiencies suppress *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B}

Deficiency	Breakpoints	*GMR-dRet*^{WT}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{MEN2B}	Candidate gene, alleles tested	Interaction
Df(2L)net-PMF	21A1;21B8	WS(100)	WS(86)	WS(83)	*kismet*: *kis*^{k10237}, *kis*^{k13416}	WS
Df(2L)al	21B8;21D1	SS(100)	MS(100)	SS(100)	*ebi*: *ebi*^{k16213}	WS
Df(2L)TW161	38A6;40B1	WS(75)	SS(96)	SS(100)	*diaphanous*[a]: *dia*^{k07135}	N
					dRet	—
Df(2R)X58-12	58D1;59A	MS(80)	SS(100)	SS(100)	*plexus*: *px*^{k08316}	WS
Df(2R)59AD	59A1;59D4	WS(78)	SS(100)	SS(100)		—
Df(3L)Pc-2q	78C5;79A1	MS(100)	WS(100)	WS(100)	*SAK*[b]: *SAK*^{c06612}	N
Df(3R)ME15	81F3;82F7	MS(90)	SS(97)	SS(100)	*Gelsolin*[c]	—
Df(3R)Antp17	84A5;84D14	WS(67)	SS(100)	MS(100)		—
Df(3R)Hu	84A6;84B6, 84D4;84F2	WS(76)	MS(100)	MS(82)		—
Df(3R)by10	85D8;85F1	nt	WS(97)	WS(77)	*Ras85D*: *Ras85D*^{06677}, *Ras85D*$^{\Delta c40b}$	WS-MS
Df(3R)DG2	89E1;91B2	SS(100)	SS(100)	SS(100)	*glass*: *gl*^{2}	SS
Df(3R)Cha7	90F1;91F5	WS (89)	WS(100)	WS(100)	*glass*: *gl*^{2}	SS

Each deficiency is listed by name. The cytological breakpoints for each are derived from the FlyBase and Bloomington Stock Center online databases. Genetic interactions are indicate according to strength of phenotype: W, weak; M, moderate; S, strong; and type of interaction: S, suppressor; N, no interaction; nt, not tested. Therefore, SS is a strong suppressor. Parentheses indicate the penetrance of the interaction calculated as a percentage. See text for details on candidate genes. —, indicates that there were no mutants in the candidate gene available for testing.

[a] *dia* encodes a formin homology protein involved in cytoskeleton remodeling and Rho signaling (Prokopenko *et al.* 1999).

[b] Encodes a serine/threonine kinase, which is a putative effector of Src signaling (Yamashita *et al.* 2001).

[c] *Gelsolin* encodes an actin-binding protein linked to Src signaling (Chellaiah *et al.* 2000).

TABLE 3

Deficiencies enhance *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B}

Deficiency	Breakpoints	*GMR-dRet*^{WT}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{MEN2B}	Candidate gene, alleles tested	Interaction
Df(1)C128	7D1;7D5	SE(100)	SE(100)	SE(100)	*myospheroid*[a]: *mys*^{1}	N
Df(1)RA2	7D18;8A4	SE(100)	ME(100)	ME(100)	*Neuroglian*: *Nrg*^{14}, *Nrg*^{17}	WE
Df(1)KA14	7F1;8C6	WE(100)	WE(100)	ME(100)	*Neuroglian*: *Nrg*^{14}, *Nrg*^{17}	WE
Df(1)lz-90b24	8B5;8D9	SE(100)	WE(45)	SE(100)	*Moesin: Moe*^{G0404}, *Moe*^{G0415}	WE
Df(1)sd72b	13F1;14B1	WE(78)	WE(26)	ME(100)	*vap(RasGAP)*	—
Df(1)B25	15D3;16A6	WE(67)	WE(62)	WE(90)		—
In(2LR)DTD16^{L}*DTD42*^{R}	23C;23E6	WE(68)	SE(100)	ME(84)	*Chd1*[b]	—
Df(2L)cact-255rv64	35F;36D	SE(88)	ME(92)	SE(100)	*cadN2*: *cadN2*^{BG02611a}	WE
Df(2L)H20	36A8;36F1	ME(100)	SE(90)	ME(100)	*cadN2*: *cadN2*^{BG02611a}	WE
Df(2L)TW137	36C2;37B10	SE(89)	SE(100)	ME(100)	*cadN2*: *cadN2*^{BG02611a}	WE
Df(2L)TW50	36E4;38A7	WE(86)	SE(100)	ME(91)	*Paxillin, Pax*^{EY02020}	WE
Df(2R)vg-C	49B2;49E2	SE(85)	SE(98)	SE(96)	*Sin3A*: *Sin3A*^{08269}, *Sin3A*^{ex4}	WE
Df(3R)T-32	86C1;87B5	WE(100)	WE (58)	WE(93)	*dCsk*: *dCsk*^{j1D8}	WE
Df(3R)M-Kxl	86E2;87C7	WE(88)	ME(79)	WE(79)	*dCsk*: *dCsk*^{j1D8}	WE
Df(3R)e-N19	93B2;94A8	WE(78)	SE(96)	WE	*Rab11*[c]: *Rab11*^{93Bi}, *Rab11*^{j2D1}	N
					cortactin[d]	—
					RhoGAP93B[e]:*RhoGAP93B*^{EY06358}	N
Df(3R)e-R1	93B6;93D4	WE(54)	SE(100)	ME(100)	*Rab11*[c]: *Rab11*^{93Bi}, *Rab11*^{j2D1}	N
					cortactin[d]	—
					RhoGAP93B[e]:*RhoGAP93B*^{EY07163}	N

Each deficiency is listed by name. The cytological breakpoints for each are derived from the FlyBase online database and the Bloomington Stock Center. Genetic interactions are indicated according to strength of phenotype: M, moderate; S, strong; W, weak; and type of interaction: S, suppressor; E, enhancer; N, no interaction. Therefore, SS is a strong suppressor. Parentheses indicate penetrance of the interaction calculated as a percentage. —, indicates that there were no mutants in the candidate gene available for testing.

[a] *mys* encodes a PS integrin β-subunit, which are proteins that regulate focal adhesions and Src signaling (Brown 1994).

[b] *Chd1* encodes a chromatin-remodeling protein very similar to dMi-2 (Stokes *et al.* 1996).

[c] *Rab11* encodes a small GTPase that regulates endosomal trafficking of transmembrane receptors (Cullis *et al.* 2002).

[d] *cortactin* encodes a known substrate for Src kinases (Wu *et al.* 1991; Okamura and Resh 1995).

[e] *RhoGap93B* is an ortholog of RhoGAP, which itself is a regulator of Ret^{MEN2} activity (Chiariello *et al.* 1998).

Deficiency screens: enhancers of *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B}: Sixteen deficiencies that define 11 genomic regions were isolated as enhancers of *GMR-dRet* (Table 3). Again, some of these deficiencies remove genes that are known negative regulators of RTKs and the Ras pathway (Table 3). The enhancing deficiencies *Df(1)RA2* and *Df(1)KA14* together define a region, 7F1;8A4, that includes *Neuroglian* (*Nrg*). In the developing eye, *Nrg* negatively regulates dEGFR activity (Islam *et al.* 2003). Reducing *Nrg* gene dosage enhanced *GMR-dRet* activity, indicating that it also can negatively regulate *dRet* activity (Table 3). *Df(2R)vg-C* (49B2;49E2) behaved as a strong enhancer of *GMR-dRet*^{WT}, *GMR-dRet*^{MEN2A}, and *GMR-dRet*^{MEN2B} (Table 3). *Sin3A*, which also enhanced all three forms of *dRet*, maps to this interval and encodes a chromatin-remodeling factor that is a known regulator of the Ras pathway (Neufeld *et al.* 1998; Rebay *et al.* 2000).

Enhancer regions that contained regulators of Src signaling were also recovered. For example, two overlapping deficiencies, *Df(3R)T-32* and *Df(3R)M-Kxl*, define 86E2;87B5. Smaller overlapping deficiencies (Kusano *et al.* 2001) were used to more finely map this region: *Df(3R)T-61* (86E3;87A9) and *Df(3R)pros235* (86E2;86F4) enhanced *GMR-dRet*^{MEN2B}, whereas *Df(3R)pros640* (86E2;86E11) and *Df(3R)T7* (86F1;86F4) failed to modify the *GMR-dRet*^{MEN2B} phenotype, identifying an enhancer region within 86E11;86F1. A strong candidate that maps to 86E17 is *dCsk*, which encodes the Drosophila C-terminal Src kinase ortholog (Read *et al.* 2004). A strong hypomorphic mutation in *dCsk* enhanced *GMR-dRet*^{MEN2B} and *GMR-dRet*^{WT}. Mutations that reduce *dCsk* function lead to hyperactivation of dSrc activity (Read *et al.* 2004), suggesting that dRet acts through Src. Other modifier deficiencies also deleted regulators of Src signaling (Tables 2 and 3). Unfortunately, mutations in many of these genes were not available, but as more Drosophila genes are mutated, these candidates and others can be tested.

Disregulation of cell adhesion contributes to tumorigenesis and enhancing deficiencies identified multiple regions that contain regulators of cell adhesion: *Df(1)lz-90b24* (8B5;8D9) uncovers a locus encoding the Moesin ortholog Moe (Speck *et al.* 2003); *Df(2L)TW50* (36E04-F01;38A06-07) defines a region that includes Drosophila Paxillin, a component of focal adhesions; and a series of enhancing deficiencies [*Df(2L)TW50*, *Df(2L)H20*, and *Df(2L)TW137*] define two genomic regions, 36C2;

TABLE 4

Deficiencies that differentially interact with *GMR-dRet^MEN2A^*

Deficiency	Breakpoints	*GMR-dRet^WT^*	*GMR-dRet^MEN2A^*	*GMR-dRet^MEN2B^*
Df(2R)ST1	42B3;43E18	WS(15)	N	WS(88)
Df(2R)CX1	49C1;50D2	WS(48)	WE(40)	WS(93)
Df(2R)P34	55E2;56C11	WS(10)	WE(24)	MS(87)
Df(2R) AA21	56F9;57D12	WS(67)	N	MS(98)
Df(2R)Pu-D17	57B4;58B	SS(97)	N	MS(100)
Df(3L)ZN47	64C;65C	SE(100)	N	SE(100)
Df(3R)Dl-BX12	91F1;92D6	N	WS(89)	WS(17)

Each deficiency is listed by name. Breakpoints are derived from the FlyBase online database and the Bloomington Stock Center. Genetic interactions are indicated according to strength of the phenotype: W, weak; M, moderate; S, strong; and type of interaction: S, suppressor; E, enhancer; N, no interaction. For example, WS is a weak suppressor. Parentheses indicate the penetrance of the interaction calculated as a percentage.

36D and 36E4;36F1, one of which contains *cadN2*, an N-cadherin ortholog, which mediates adherins junctions and regulates JNK signaling (Charrasse *et al.* 2002). Mutations in *Moe*, *Paxillin*, and *cadN2* all enhanced *GMR-dRet* isoforms (Table 3). Finally, not all deficiencies had clear candidate modifiers, highlighting the difficulties in using chromosomal deficiencies to identify truly novel modifiers.

Modifiers that may distinguish *GMR-dRet^MEN2A^* from *GMR-dRet^WT^* and *GMR-dRet^MEN2B^*: Ret^MEN2B^ has been proposed to signal through pathways not targeted by Ret^MEN2A^ or normal Ret signaling. However, no deficiency modifiers clearly distinguished *GMR-dRet^MEN2B^* activity from that of *GMR-dRet^MEN2A^* and *GMR-dRet^WT^* in our screen. Surprisingly, seven deficiencies distinguished *dRet^MEN2A^* from *dRet^MEN2B^* and *dRet^WT^* (Table 4). Most of these showed weak interactions with one *dRet* isoform but not another; these data may simply reflect an inability to unambiguously score weak interactions. However, two deficiencies enhanced *dRet^MEN2A^* but suppressed *dRet* and *dRet^MEN2B^* (Table 4). The C695R MEN2A mutation likely causes constitutive dimerization of the dRet extracellular domain, and perhaps these two deficiencies remove loci that regulate this process to affect dRet^MEN2A^ in a unique manner. However, we did not finely map these regions or identify any corresponding loci for these deficiencies.

***P*-element screen:** As a companion to the candidate gene and deficiency screens, *P*-element lines were similarly screened for *GMR-dRet* modifiers. As part of a large-scale gene disruption project, the Berkeley *Drosophila* Genome Project (BDGP) has collected thousands of lethal mutations associated with stable insertions of modified *P*-element transposons (Deak *et al.* 1997; Spradling *et al.* 1999). These collections, available from the Drosophila stock centers, contained >2000 individual disrupted genes at the time of our screen. The purpose of screening *P*-element lines was to identify unexpected genes that dominantly modified *GMR-dRet*. Such genes may represent novel regulators of Ret-dependent oncogenesis. This screen allowed us to expand from the candidate approach inherent in the deficiency screen to take an unbiased functional genomics approach.

*GMR-dRet^MEN2B^* flies were crossed to flies heterozygous for 2524 separate *P*-element mutations. For this screen we utilized flies from lines *GMR-dRet^MEN2B^-3B* and *GMR-dRet^MEN2B^-3C*, which carried single transgenes that confer intermediate phenotypes; these lines were especially sensitive to mutations in known RTK effectors. The screen design was similar to that of our comparative deficiency screen (Figure 5). Again, each cross gave two classes of F_1 progeny that were compared to each other. Enhancers and suppressors were rescreened twice to confirm genetic interactions and to determine penetrance. A total of 90 initial *P*-element modifiers were recovered. These initial modifiers were then put through a series of genetic tests to remove nonspecific modifiers and to confirm specific modifier loci.

Secondary screens to eliminate nonspecific GMR-dRet^MEN2B^ modifiers: We employed several secondary tests to eliminate modifiers that altered *GMR-dRet^MEN2B^* phenotypes for reasons other than their effects on dRet^MEN2B^. Eleven enhancers exhibited dominant rough-eye phenotypes even in the absence of *GMR-dRet^MEN2B^* constructs; these were discarded. Modifiers were screened with additional *GMR-dRet^MEN2B^* lines to determine if genetic interactions were line specific. Two modifiers interacted with only one *GMR-dRet^MEN2B^* line: these were discarded since they may alter *GMR-dRet^MEN2B^* phenotypes due to site-specific insertion effects. To eliminate modifiers that acted on the *GMR* promoter, all confirmed modifiers were tested with multiple unrelated *GMR*-containing transgenes that also cause a dominant rough-eye phenotype (supplemental Table S1 at http://www.genetics.org/supplemental/); five modifiers were eliminated by these tests. Together, these tests left 72 modifiers for further study.

Secondary screens for genetic verification of GMR-dRet modifiers: Many *P*-element lines harbor additional mutations not associated with the P insertion (Spradling *et al.* 1999). Therefore, we attempted to verify that the

genes disrupted by *P*-element insertions were indeed *GMR-dRet* modifiers. Some *P*-element lines were outcrossed to remove second-site mutations by recombination and retested with $GMR\text{-}dRet^{MEN2B}$ (supplemental Table S2 at http://www.genetics.org/supplemental/); three lines lost their modifier activity upon out-crossing and were discarded. As another approach, we mobilized the *P*-element insertion in several lines using P transposase: "clean" excision of the *P* element out of the locus should revert modifier activity if it is due to *P*-element disruption (supplemental Table S2). For three lines, excision did not revert modifier activity, even in cases where excision reverted the insertion-associated lethality. Second-site mutations were the likely *GMR-dRet* modifiers in these three lines. Sometimes excision did not revert the lethality of the affected chromosome, suggesting that either the excision process failed to restore the disrupted gene or the chromosome carried second-site lethal mutations; data from such lines were inconclusive. These tests left 66 lines.

Secondary screens with $GMR\text{-}dRet^{MEN2A}$ and $GMR\text{-}dRet^{WT}$: The purpose of these secondary screens was to distinguish between modifiers common to all *dRet* isoforms and $dRet^{MEN2B}$-specific modifiers. Factors common to $GMR\text{-}dRet^{MEN2B}$ and $GMR\text{-}dRet^{MEN2A}$ were of particular interest since such factors may represent genes that are important for Ret^{MEN2}-mediated tumorigenesis. Our phenotypic data and deficiency screen suggested that all three isoforms of *dRet* were largely equivalent. We further tested this idea by screening $GMR\text{-}dRet^{MEN2B}$ modifiers for their ability to modify $GMR\text{-}dRet^{WT}$ and $GMR\text{-}dRet^{MEN2A}$ flies (Table 5). All $GMR\text{-}dRet^{MEN2B}$ modifiers tested genetically interact with $GMR\text{-}dRet^{MEN2A}$ and/or $GMR\text{-}dRet^{WT}$ (Table 5), again suggesting that all three dRet isoforms rely on the same pathways. Interestingly, $GMR\text{-}dRet^{MEN2A}$ insertions that had strong phenotypes frequently showed weak or no interactions with weak enhancers of $GMR\text{-}dRet^{MEN2B}$, although insertions of $GMR\text{-}dRet^{MEN2A}$ that gave rise to milder phenotypes did interact with most of these weak enhancers. This suggests that $dRet^{MEN2A}$ is less sensitive than $dRet^{MEN2B}$ to alterations in gene dosage of downstream targets, an observation that may explain the failure of some deficiencies to interact with $dRet^{MEN2A}$ (see above).

Secondary screens with $dEGFR^{Elp}$: To identify $dRet^{MEN2B}$ modifiers that are also general regulators of RTKs, we rescreened them using $dEGFR^{Elp}$, which encodes an activated allele of the ortholog dEGFR. $dEGFR^{Elp}/+$ flies have a dominant rough-eye phenotype caused by ectopic dEGFR activity (Lesokhin *et al.* 1999). All $GMR\text{-}dRet^{MEN2B}$ modifiers were crossed to $dEGFR^{Elp}$ to determine their ability to modify an independent RTK (Table 5). As expected, members of the *Ras* pathway modified $dEGFR^{Elp}$. Surprisingly, many $GMR\text{-}dRet^{MEN2B}$ modifiers failed to genetically interact with $dEGFR^{Elp}$, suggesting that $dRet^{MEN2B}$ may act through a subset of genes not utilized by dEGFR.

Molecular identification, verification, and function of *GMR-dRet* modifier loci: The major advantage to screening *P*-element lines is that the transposon provides a molecular tag for identifying the disrupted locus. To help associate *P*-element modifier lines with corresponding disrupted genes, we sequenced genomic DNA flanking the transposon insertion sites. Concomitantly, other groups, including BDGP, also generated sequences for many lines. Genomic regions flanking *P*-element insertions were mapped to the full Drosophila genome sequence. The surrounding sequences were searched for known genes, matching ESTs, and predicted ORFs using the FlyBase Genome and Gadfly browsers and annotated GenBank files. To date, flanking genomic sequence has been obtained and analyzed for 34 *P*-element modifiers (supplemental Table S3 at http://www.genetics.org/supplemental/). A total of 33 yielded sequence that indicated that the P inserted in or near a gene. Other groups have characterized an additional six *P*-element modifiers and identified the disrupted genes (Table 4) (Salzberg *et al.* 1997; Spradling *et al.* 1999). Thus, we were able to associate 40 *P*-element modifier lines with disrupted genes, which were candidate modifier loci.

On the basis of previous studies, sequence data, and our own complementation tests, we identified additional alleles for many modifiers and tested their ability to modify our *GMR-dRet* constructs (Table 6). If the ability of a *P*-element line to genetically modify $GMR\text{-}dRet^{MEN2B}$ is due to disruption of a gene associated with the insertion, then other mutant alleles or matching deficiencies for that gene should show similar modifier activity. We concentrated on testing null alleles whenever possible. Additional alleles of five *P*-element lines failed to modify *GMR-dRet* phenotypes: these lines probably contain second-site mutations that modified $GMR\text{-}dRet^{MEN2B}$ (Table 6). In other cases, no additional alleles were available or problems with the strength of available alleles made such tests inconclusive (Table 6; data not shown). Importantly, we verified 12 genes from 16 *P*-element lines as *GMR-dRet* modifiers because multiple alleles or matching deficiencies for these genes proved to have similar modifier activity (Table 6). These verified loci were *misshapen*, *Ras85D*, *drk*, *kismet*, *ebi*, *Sin3A*, *dMi-2*, *spitz*, *Star*, *Delta*, *hedgehog*, and *dCsk*. We further investigated the functions of some of these loci.

Ras pathway and related loci: As expected, we identified members of the Ras signal transduction pathway as modifiers of *GMR-dRet* (Table 6). These include mutations in canonical pathway members such as *drk*, a Grb2 ortholog, and *Ras85D* in addition to other regulators such as *spitz*, *Star*, *kismet*, and *ebi*. Mutations that affect signaling through the dEGFR pathway also interacted with dRet. These include mutations in *spitz*, a ligand for dEGFR, and *Star*, a protein required for proper processing of Spitz protein (Lee *et al.* 2001; Urban *et al.* 2002). Note that although *Star* mutations sometimes give

TABLE 5

Genetic interactions between $GMR\text{-}dRet^{MEN2B}$ *P*-element modifiers and $GMR\text{-}dRet^{WT}$, $GMR\text{-}dRet^{MEN2A}$, and $dEGFR^{Elp}$

Gene/allele	$GMR\text{-}dRet^{MEN2B}$	$GMR\text{-}dRet^{MEN2A}$	$GMR\text{-}dRet^{WT}$	MEN2B specific	$dEGFR^{Elp}$
msn^{j1E2}	ME(88)	WE(18)	WE(50)	No	WE
$dCsk^{j1D8}$	WE(76)	N	WE(52)	No	N
hh^{rJ413}	WE(84)	WE(92)	ME(100)	No	SE
crb^{j1B5}	WE(93)	WE(69)	WE(100)	No	N
l(3)j2D5	ME(95)	WE(76)	WE(82)	No	WS
drk^{k02401}	SS(95)	N	N?	No[a]	WS
spi^{s3547}	WS(86)	WS(65)		No	ME[b]
px^{k08316}	WS(73)	N	WS(54)	No	WE
S^{k09538}	WS(67)	WS(14)	N	No[a]	ME[b]
$kismet^{k10237}$	WS(80)	WS(71)	N	No	ND
drk^{k13809}	WS(100)	N	WS(23)	No	N
ebi^{k16213}	SS(85)	WS(65)	WS(18)	No	N
$Ras85D^{06677}$	WS(76)	MS(100)		No	ND
l(3)06803	WS(95)	MS(91)		No	WE
l(3)06906	WS(100)	MS(100)		No	N
l(3)j5B6	WE(97)	ME(100)		No	N
l(3)j4B9	WE(95)	WE(68)		No	N
$neur^{j6B12}$	WE(94)	WE(47)		No	N
$Pp1\text{-}87B^{j6E7}$	WE(85)	WE(29)		No	WE
$scrib^{j7B3}$	WE(98)	WE(45)		No	ME
$Sin3A^{08269}$	WE(86)	WE(100)	WE(77)	No	N
drk^{10626}	SS(100)	WS(24)	MS(100)	No	N
l(3)S005504	ME(91)	WE(30)	WE(70)	No	N
l(3)S001405	WE(95)	N	WE(58)	No	WE
l(3)S003704	WE(94)	N		ND	WE
l(3)S000718	WE(96)	N	WE(47)	No	N
l(3)S009515	WE(93)	N	WE(62)	No	N
l(3)S000710	WE(82)	WE(26)		No	N
l(3)S012805	WE(100)	N	WE(97)	No	N
l(3)S016805	WE(88)	WE(12)	WE(96)	No	N
l(3)S023708	ME(94)	WE(15)	N	No	N
l(3)S023549	WE(82)	WE(50)		No	N
l(3)S024833	WE(96)	WE(33)		No	N
l(3)S023901	WE(100)	WS(39)	WE(79)	No	N
l(3)S022231	WE(91)	N	WE(80)	No	N
l(3)S024503	WE(92)	N		ND	N
l(3)S026238	WE(100)	N	WE(65)	No	N
l(3)S026421	WE(94)	N	WE(90)	No	N
l(3)S024329	WE(88)	WE(71)	WE(52)	No	N
l(3)S049706	ME(85)	N	WE(82)	No	ND
l(3)S054513	WE(79)	N		ND	N
l(3)S046604	WE(96)	WE(52)	ME(95)	No	N
l(3)S049902	WE(93)	N	WE(35)	No	WE
$Dl^{S049520}$	WS(85)	WS(100)		No	N
$bon^{S048706}$	WE(93)	WE(50)	WE(81)	No	WS
l(3)S047526	ME(100)	N	SE(100)	No	WE
l(3)S066607	WE(96)	WE(22)	WE(96)	No	N
l(3)S057101	WE(95)	N	WE(59)	No	WE
l(3)S068808	WE(93)	WE(32)	WE(84)	No	N
l(3)S083407	WE(90)	WE(24)		No	N
l(3)S063512	WE(81)	WE(15)	WE(75)	No	ME
l(3)S056113	WE(88)	N	WE(50)	No	N
l(3)S090101	WE(100)	WE(38)	WE(81)	No	N
l(3)S090114	WE(79)	N	WE(76)	No	N
l(3)S141715	WE(84)	N		ND	N
l(3)S136603	WE(97)	N	WE(90)	No	N
l(3)S092708	WE(94)	N	WE(83)	No	N
l(3)S003003	WE(82)	N	WE(100)	No	WE

(*continued*)

TABLE 5

(Continued)

Gene/allele	*GMR-dRet*^{MEN2B}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{WT}	MEN2B specific	*dEGFR*^{Elp}
l(3)S050116	WE(97)	WE(49)	WE(46)	No	N
l(3)S142909	WE(84)	N	WE(85)	No	N
l(3)S147412	WE(91)	WE(39)	WE(85)	No	N
l(3)S145911	WE(91)	N	WE(69)	No	N
l(3)S146006	WS(100)	MS(100)	WS(52)	No	N
l(3)S135703	WE(86)	N	WE(81)	No	N
l(3)S133117	WE(85)	WE(45)	WE(81)	No	N
l(3)S130910	WE(85)	WS(30)	WE(40)	No	WE

Each *GMR-dRet*^{MEN2B} modifier was crossed to *GMR-dRet*^{WT} and/or *GMR-dRet*^{MEN2A} constructs to determine the specificity of *dRet*^{MEN2} genetic interactions. Not all *P*-element lines could be tested since some lines died and were no longer available from the stock centers: these are indicated as ND (not determined).

[a] Other alleles of *Star* and *drk* interact with *GMR-Ret*^{MEN2A} (Table 6). This is probably due to the hypomorphic nature of the alleles isolated in the screen.

[b] Some positive regulators of the Ras pathway such as *spitz* actually dominantly enhanced *dEGFR*^{Elp}, demonstrating that *dEGFR*^{Elp} does not show simple linear interactions with Ras-ERK kinase pathway components. This suggests that *dEGFR*^{Elp} does not constitute a simple gain-of-function model for RTK signaling; this may reflect negative feedback loops that regulate dEGFR signaling (Spencer and Cagan 2003).

dominant rough-eye phenotypes, the *Star* mutation isolated in this screen had no phenotype on its own and, in fact, suppressed *GMR-dRet* rough-eye phenotypes. Mutations in *kismet*, which encodes an ortholog of chromatin-remodeling factors and is linked to dEGFR activity (Therrien *et al.* 2000), and *ebi*, a chromatin-remodeling protein and nuclear regulator of both *Ras* and *Notch* signaling (Dong *et al.* 1999; Tsuda *et al.* 2002), also modified *GMR-dRet* phenotypes. In addition, *plexus*, which encodes a nuclear factor, has also been implicated in RTK signaling (Matakatsu *et al.* 1999). The ability of dEGFR-specific regulators to modify *GMR-dRet* suggests either that these factors may also regulate the dRet receptor on some level or that genetic reduction of signaling through a parallel RTK may alleviate the effects of dRet.

misshapen: Mutations in *misshapen* (*msn*), which encodes a Ste20 serine/threonine kinase, enhanced *GMR-dRet* (Table 6), suggesting that *msn* acts as a negative regulator of *dRet*. *msn* also enhanced *dEGFR*^{Elp}. Msn activity is required for cytoskeletal remodeling and cell shape change during development in processes such as axon pathfinding and dorsal closure (Treisman *et al.* 1997; Su *et al.* 1998; Ruan *et al.* 1999) and can also act as a negative regulator of Ras pathway signaling (Huang and Rubin 2000). Msn functions in at least two separate signaling pathways: downstream of *dock*, the Drosophila ortholog of the Nck adaptor protein, and upstream of *bsk*, the Drosophila ortholog of JNK (Su *et al.* 1998; Paricio *et al.* 1999; Ruan *et al.* 1999, 2002). Mutations in *dock* did not modify *GMR-dRet*^{MEN2}. Mutations in *bsk* did modify *GMR-dRet*^{MEN2B}, although *bsk* mutations acted as suppressors (Table 1). Thus, *bsk* and *msn* modified *GMR-dRet* in opposite directions, suggesting either that *msn* and *bsk* are not in a simple linear pathway or that, in response to dRet, *msn* acts in a pathway that is separate from *dock* or *bsk*.

Hedgehog and Delta-Notch signaling pathways: Mutations in *Delta*, which encodes a membrane-bound ligand of the Notch receptor, were found to be suppressors of *GMR-dRet* (Table 6). This interaction suggests a functional connection between Notch signaling and *dRet* activity. Connections between Notch and Ras pathway signaling have previously been noted: for example, mutations in *Delta* modify Ras signaling (Maixner *et al.* 1998), *Delta* expression is regulated by *Ras* activity in the eye (Tsuda *et al.* 2002), and *Notch* and *Ras* signaling act together in a number of developmental models (*e.g.*, Baker and Rubin 1992; Miller and Cagan 1998; Carmena *et al.* 2002). Mutations in *hedgehog* (*hh*), which encodes a secreted activator of the Hh/Smo/Ptc signal transduction pathway, were also found to modify *GMR-dRet* signaling. Hh is expressed in the larval eye epithelium by maturing ommatidia and directs proper progression of the morphogenetic furrow (Heberlein *et al.* 1995); its modification of *GMR-dRet* may reflect disruption of furrow progression by aberrant *dRet*-expressing ommatidia. Alternatively, Hh is also known to modify Ras85D function (Karim *et al.* 1996), and it may modify *dRet* function through this capacity.

dMi-2, Sin3A, and histone deacetylase complexes: We also identified components of the SIN3 and NuRD histone deacetylase complexes (reviewed in Ahringer 2000), a category of genes that has not been previously linked to Ret. For example, multiple alleles of *Sin3A* acted as *GMR-dRet* enhancers (Table 6). Sin3A, a part of the SIN3 complex, is a transcriptional corepressor (Neufeld *et al.* 1998; Pennetta and Pauli 1998). *Sin3A* mutations also dominantly enhance eye phenotypes caused by overexpression of the Yan transcriptional repressor (Rebay *et al.* 2000) and dominantly suppress Sina overexpression eye phenotypes (Neufeld *et al.* 1998); perhaps significantly, both nuclear factors are downstream

TABLE 6

Genetic interactions between *GMR-dRet* isoforms and additional alleles of candidate *P*-element modifiers

Gene	Gene function	Allele or deficiency	*GMR-dRet*^{MEN2B}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{WT}	Comments	Decision
misshapen	Ste20 serine/threonine kinase	*msn*^{j1E2}	ME	WE(18)	WE(50)		Modifier
		msn^{03349}	ME(61)				
dCsk	C-terminal Src kinase	*dCsk*^{j1D8}	WE	N	WE(52)	*dCsk*^{j1D8} is a strong hypomorph; deficiencies pinpoint *dCsk* region.	Modifier
		Df(3R)M-Kxl	WE(79)	ME(79)	WE(88)		
		Df(3R)T-32	WE(93)	WE(58)	WE(100)		
		Df(3R)T-61	WE (80)				
hedgehog	Secreted ligand in Hedgehog pathway	*hh*^{rJ413}	WE	WE(92)	ME(100)	*hh*^{AC} is null; *hh*^{2} and *hh*^{21} are hypomorphs.	Modifier
		hh^{neo56}	WS(32)				
		hh^{AC}	WE(24)				
		hh^{2}	WE(15)	WE(73)			
		hh^{21}	N				
drk	Ortholog of Grb2	*drk*^{k02401}	SS	N	N	*drk*^{10626} is the strongest allele; none are true nulls.	Modifier
		drk^{k13809}	WS	N	WS(23)		
		drk^{10626}	SS	WS(24)	MS(100)		
spitz	dEGFR ligand	*spi*^{s3547}	WS	WS(65)			Modifier
		spi^{01068}	WS(46)				
		spi^{1}	WS(56)	WS(40)			
plexus	Nuclear factor involved in Ras pathway	*px*^{k08316}	WS	N	WS(54)	*px*^{k08134} is a weak hypomorph.	Unclear
		px^{k08134}	N				
		Df(2R)X58-12	SS(100)	SS(100)	MS(80)		
Star	Protein required to process and activate *spitz*	*S*^{k09538}	WS	WS(14)	N		Modifier
		S^{IIN}	WS(42)	WS(25)			
		S^{k09530}	WS(63)	N			
kismet	Chromodomain protein involved in Ras pathway	*l(2)k10237*	WS	WS(71)	N	Noncomplementation with known *kismet* alleles confirms that *l(2)k10237* and *l(2)k16510* are *kismet* mutations.	Modifier
		l(2)k16510	WS(36)				
		kis^{k13416}	WS(79)		N		
		Df(2L)net-PMF	WS(100)	WS(86)	WS(83)		
ebi	Nuclear protein involved in Ras pathway	*ebi*^{k16213}	SS	WS(65)	WS(18)		Modifier
		Df(2L)al	SS(100)	MS(100)	SS(100)		
Ras85D	Ras ortholog	*Ras85D*^{06677}	WS	MS(100)		*Ras85D*$^{\Delta c40b}$ is a null.	Modifier
		Ras85D$^{\Delta c40b}$	WS				
Pp1-87B	Phosphatase	*Ppi-87B*^{j6E7}	WE	WE(29)			Unclear
		Pp1-87^{B1}	WE(10)	N	WE(24)		
		Pp1-87^{B2}	WE(29)	WS(47)			
		Df(3R)ry615	WE(17)				
Sin3A	Transcriptional corepressor	*Sin3A*^{08269}	WE	WE(100)	WE(77)	*Sin3A*^{ex4} is a null.	Modifier
		Sin3A^{HW52}	ME(98)				
		Sin3A^{k07401}	ME(100)				

(*continued*)

TABLE 6

(Continued)

Gene	Gene function	Allele or deficiency	*GMR-dRet*^{MEN2B}	*GMR-dRet*^{MEN2A}	*GMR-dRet*^{WT}	Comments	Decision
		Sin3A^{k08919}	SE(82)		ME(85)		
		Sin3A^{ex4}	ME(93)	WE(52)	ME(97)		
		Df(2R)vg-C	SE(96)	SE(98)	SE(85)		
Mi-2	Chromodomain protein and ATP-dependent helicase	*l(3)S005504*	ME	WE(30)	WE(70)	Noncomplementation with *Mi-2*^{j3D4} confirms *l(3)S005504*, *l(3)S047526*, and *l(3)S147412* as *dMi-2* alleles.	Modifier
		l(3)S047526	ME	N	SE(100)		
		l(3)S147412	WE	WE(39)	WE(85)		
		Mi-2^{j3D4}	ME(52)				
Delta	Transmembrane ligand for the Notch receptor	*Dl*^{S049520}	WS	WS(100)			Modifier
		Dl^{9P}	WS(78)	MS(88)			
crumbs	Transmembrane cell adhesion receptor	*crb*^{j1B5}	WE	WE(69)	WE(100)	*crb*^{8F105} and *crb*^{2} are nulls.	False
		crb^{8F105}	WS(18)				
		crb^{2}	WE(41)	N			
		l(3)07207	WS(22)	WS(63)	WE(23)		
		Df(3R)crbF89-4	N				
scribble	Cell polarity protein	*scrib*^{j7B3}	WE	WE(45)		*scrib*^{1} and *scrib*^{2} are nulls.	False
		scrib^{1}	N	WS(59)			
		scrib^{2}	N	WS(67)			
string	Cell cycle regulator	*l(3)S024503*	WE			Complementation tests performed in Salzberg *et al.* (1997)	False
		l(3)S022406	N				
		l(3)S043922	N				
		l(3)S073013	N				
		l(3)S089605	N				
		stg^{01235}	N				
		Df(3R)3450	N				
neuralized	Ubiquitin ligase	*neur*^{j6B12}	WE	WE(23)		*neur*^{1} is a strong hypomorph; *neur*^{11} is a null.	False
		neur^{11}	N	N	WE(29)		
		neur^{1}	N				
		neur^{3}			N		
		neur^{A101}	WS(24)	WS(79)			
		neur^{neo37}	N				
cyclin A	Cyclin A ortholog	*l(3)S054513*	WE			Complementation tests performed by Salzberg *et al.* (1997)	False
		l(3)S003302	N				
		l(3)S004024	N				
		l(3)S004623	N				
		l(3)S010407	N				
		l(3)S024534	N				
		l(3)S052106	N				
		l(3)S144905	N				
		CycA^{03946}	N				

Allelic strength is generally noted in the FlyBase online database based on published literature. Candidates were called "modifier" if more than an additional allele or matching deficiencies showed reproducible and highly penetrant genetic interactions with one or more form of *GMR-dRet*. Candidates were called "false" if null alleles failed to interact. Many additional alleles showed low penetrance interactions, making the results "unclear."

effectors of Ras signaling. Moreover, *Sin3A* may be a direct transcriptional target of Ras signaling (Asha *et al.* 2003).

Three alleles of *dMi-2* enhanced *GMR-dRet* (Table 6). A member of the NuRD complex, *dMi-2* encodes an ortholog of Mi-2, an ATP-dependent DNA helicase that contains multiple protein-protein and DNA interaction domains (Kehle *et al.* 1998). Components of the NuRD complex have been strongly implicated in human cancers (Brehm *et al.* 2000; Fujita *et al.* 2003). Mutations in *dMi-2* also enhanced the effect of p21 overexpression within the retina (supplemental Table S1 at http://www.genetics.org/supplemental/), linking *dMi-2* to the cell cycle. *dMi-2* mutations have not been previously isolated in screens for regulators of RTK signaling or cell cycle. In *Caenorhabditis elegans*, the NuRD complex specifically antagonizes Ras signaling during vulval development possibly through repression of Ras pathway target genes (Solari *et al.* 1999; von Zelewsky *et al.* 2000; Chen and Han 2001). We found that mutations in *dMi-2* genetically enhanced rough-eye phenotypes caused by overexpressing the Ras85D-activated isoform Ras85D^{V12} in the retina (data not shown), illustrating that *dMi-2* may negatively regulate the Ras pathway in Drosophila as well. Also in support of this link, Ttk, a transcription factor that negatively regulates the Ras pathway, has been shown to bind and colocalize with *dMi-2 in vivo* (Lai *et al.* 1996; Murawsky *et al.* 2001).

To determine if other members of the NuRD complex influence dRet^{MEN2} function, we tested mutations in *Rpd3*, which encodes a histone deacetylase that is a NuRD component. Removing a single copy of *Rpd3* had no effect on *GMR-dRet^{MEN2}* phenotypes (data not shown). To determine if *dMi-2* mutations cause cellular defects similar to those seen with *dRet^{MEN2}* overexpression, *dMi-2* homozygous mutant retinal tissue was created using mitotic recombination. *dMi-2* mutant adult retinae appeared small and rough and showed degeneration of photoreceptor neurons (data not shown), indicating that *dMi-2* likely regulates a range of factors.

Src-related kinases: GMR-dRet phenotypes were dominantly suppressed by mutations in the Drosophila Src orthologs *Src42A* and *Src64B*, indicating that *dRet* function required Src activity. Further linking dRet and Src signaling, we identified an insertion in the Drosophila Csk ortholog *dCsk* as an enhancer. Csk family proteins inhibit Src activity through tyrosine phosphorylation of their C-terminal region and mutation of this Csk target site leads to upregulation of Src kinase activity (reviewed in Bjorge *et al.* 2000). We and others have recently explored the details of dCsk activity in the eye (Stewart *et al.* 2003; Read *et al.* 2004). Decreased *dCsk* function leads to increased cell proliferation driven by increased Src, Jun kinase, and STAT signaling (Read *et al.* 2004). Consistent with these interactions, the Drosophila Jun kinase ortholog, *bsk*, suppressed *GMR-dRet*. Therefore, *dCsk* mutations most likely enhanced *GMR-dRet* by causing elevated Src and Jun kinase activity. However, we were not able to confirm a role for STAT activity in dRet signaling: removal of one or both copies of the sole Drosophila STAT ortholog, *Stat92E*, did not modify the *GMR-dRet^{MEN2}* phenotype (data not shown). Therefore, *Stat92E* does not appear to be a rate-limiting factor for dRet^{MEN2} function in the Drosophila eye.

Loss-of-heterozygosity studies in human tumor tissue: One goal in this study was to assess the role human orthologs of *GMR-dRet* modifiers play in primary human tumors. If human orthologs of *GMR-dRet* modifiers are specifically involved in the Ret^{MEN2}-transformed phenotype, we might expect to find mutations in these orthologs in Ret^{MEN2}-associated neoplasms. We developed a list of functional human orthologs for select *GMR-dRet* enhancers (Table 6). In the fly, enhancers worsened the dRet^{MEN2}-transformed phenotype when just one copy of the gene was removed, implicating the human orthologs of these genes as candidate tumor suppressors.

Tumor suppressors are typically cellular recessives and, as such, loss-of-function mutations involving both alleles are common in neoplasms. Reduced gene function caused by mutation of a single allele (haploinsufficiency) for a variety of tumor suppressors can be sufficient to promote tumorigenesis (reviewed in Santarosa and Ashworth 2004). One means by which these mutations occur is via somatic mutation or chromosomal deletion of one or both alleles. MEN2 patients most commonly develop MTC and pheochromocytoma (adrenal tumors). The importance of Ret in these tumors became evident with the discovery that sporadic MTC and pheochromocytomas frequently harbor Ret mutations (Chiefari *et al.* 1998; Scurini *et al.* 1998). Both tumor types show secondary somatic chromosomal deletions, suggesting a role for tumor suppressors in tumor initiation or progression, but the identities of such genes are unknown (Khosla *et al.* 1991; Mulligan *et al.* 1993a; Marsh *et al.* 2003). In addition, pheochromocytoma is observed in only a subset of MEN2 patients, further suggesting its dependence on mutations in secondary modifier loci. Using archived tumor tissues obtained through the Washington University Multiple Endocrine Neoplasia Program, we assessed both sporadic and inherited MTC and pheochromocytoma tumor samples for LOH in the human orthologs of loci identified as enhancers in our Drosophila models.

Ten sporadic and 10 MEN2-associated MTC tumors and were evaluated for somatic deletion using intragenic CA repeat markers in nine loci (primer sequences and conditions available upon request). Tumor and normal DNA samples from each patient were examined for the presence of polymorphisms in these markers and tested for the somatic loss of those polymorphisms (LOH) in the tumor compared to control DNA. None of the nine loci tested to date showed frequent LOH in MTC (Table 7). In fact, three of the four LOH events

TABLE 7

Candidate tumor suppressors: LOH in human orthologs of *GMR-dRet*^{MEN2} enhancers

Gene	*GMR-dRet*^{MEN2B}	Human ortholog	Human location	Type of protein/ function	LOH pheos	LOH MTC
msn	E	*NIK*	2q11.2–q12	Ste20 serine/threonine kinase	1/9	0/11
		MINK	17p13.1	Ste 20 serine/threonine kinase	4/10	ND
		TNIK (underlined)	3q26.31	Ste20 serine/threonine kinase	86% (6/7)	1/11
dCsk	E	*Csk*	15q23–25	C-terminal Src kinase	1/8	0/10
hedgehog	E	*shh*	7q36	Hedgehog pathway ligand	0/7	2/13
		indian hh	2q33–q35	Hedgehog pathway ligand	1/7	0/13
		desert hh	12q12–q13	Hedgehog pathway ligand	0/6	0/11
Sin3A	E	*DKFZP434K2235*	15q23	Part of SIN3 HDAC complexes	0/11	0/7
dMi-2	E	*CHD3 (Mi-2 alpha)* (underlined)	17p13.1	Part of the NuRD HDAC complex	50% (3/6)	0/11
		CHD4 (Mi-2 beta)	12p13	Part of the NuRD HDAC complex	1/10	1/13

Human orthologs to select *GMR-dRet*^{MEN2} enhancers (E) were identified on the basis of sequence and functional similarity. Cytological map positions are listed for human orthologs. Both familial and sporadic pheochromocytoma (pheos) and MTCs were scanned for somatic deletions by LOH analysis using intragenic markers (see MATERIALS AND METHODS and text for details). In some cases, patients were not heterozygous for the markers used and these cases were not informative and are excluded from the tallies listed. Underlining indicates loci with a high rate of LOH. Three of the four allelic losses observed in MTCs were in a single tumor. ND, not determined.

seen in MTC specimens were in one tumor, which could reflect a generalized chromosomal instability in that particular MTC.

Five sporadic and seven MEN2-associated pheochromocytomas and corresponding normal DNA samples were assessed for LOH using the same intragenic CA repeat markers evaluated in MTCs (Table 7; supplemental Table S4 at http://www.genetics.org/supplemental/). Of the 10 loci tested in pheochromocytoma samples, *TNIK* showed LOH in 6 of 7 informative cases. *TNIK*, which encodes an ortholog of the Misshapen serine/threonine kinase, maps to the long arm of human chromosome 3 (3q). By typing 11 additional chromosome 3 markers, we found that the 6 pheochromocytoma samples showing LOH in *TNIK* also showed LOH in flanking markers, indicating that these tumors have large deletions that remove nearly all of 3q (supplemental Table S4). Overall, 80% of the informative pheochromocytomas had 3q LOH. Previous studies have also observed LOH of 3q in MEN2-associated pheochromocytomas and MTCs (Khosla *et al.* 1991; Mulligan *et al.* 1993a; Marsh *et al.* 2003). Of 6 informative pheochromocytomas, 3 also showed LOH of *CHD3*. *CHD3*, also known as *Mi-2*α, encodes a functional ortholog of Drosophila *dMi-2*, which maps to the short arm of chromosome 17 (17p). Using markers that span chromosome 17, we found that some of these tumors showed extensive LOH of chromosome 17 markers, indicating that these tumors harbor large deletions of the chromosome (supplemental Table S4).

Finally, to test for point mutations, small insertions, or internal deletions in *TNIK* and *CHD3*, we sequenced all but one of the exons for both genes in pheochromocytoma patient samples (data not shown; see MATERIALS AND METHODS). We used an exon-specific PCR amplification/sequencing approach for both strands as previously described (Ley *et al.* 2003). Twelve tumor/normal pairs were evaluated. The sequence analyses included patients whose tumors did not show detectable LOH at *TNIK* or *CHD3* as well as cases with LOH at these loci. In patients with LOH, no mutations in the remaining *TNIK* or *CHD3* alleles were identified. We also found no small mutations in *TNIK* and *CHD3* in patients who did not show detectable LOH. Several previously reported single-nucleotide polymorphisms were seen and comparison of the tumor and normal sequences confirmed the microsatellite marker LOH findings.

DISCUSSION

Dominant activating mutations in the Ret RTK lead to the familial MEN2 cancer syndromes. Despite identification of the causative Ret lesions, many outstanding issues remain to be addressed regarding the link between Ret hyperactivation and MEN2 diseases. For example, while nearly all individuals affected with

MEN2 develop MTC, people with these syndromes show variation in the severity of MTC, age at tumor onset, and the occurrence of other tumors (Easton *et al.* 1989; Eng *et al.* 1996; Ponder 1999). These data imply the existence of second-site mutations that modulate how different families and individuals manifest MEN2. Ret^{MEN2} mouse models show substantial variation in the penetrance of tumors depending on genetic background effects, again suggesting a requirement for secondary genetic alterations for malignant transformation by Ret^{MEN2} (Michiels *et al.* 1997; Acton *et al.* 2000; Kawai *et al.* 2000; Smith-Hicks *et al.* 2000; Cranston and Ponder 2003).

In the fly, overexpressing $dRet^{MEN2}$ isoforms induced defects associated with human Ret^{MEN2}, including Ras pathway hyperactivation, excess proliferation, and aberrant neuronal differentiation. This demonstrates that the *GMR-dRet*^{MEN2} flies constitute a useful model for Ret^{MEN2}, although our model does not duplicate all events that occur in MEN2. No doubt there are some species-specific and tissue-specific differences in Ret signaling that our approach missed. Despite these caveats, we chose the Drosophila eye due to its many advantages. Several signaling systems including RTKs have been studied in remarkable depth in the eye (reviewed in Voas and Rebay 2004). In fact, studies of Drosophila retinal development led to the initial identification of numerous components of RTK-Ras signaling pathways, including Sos, Ksr, and Cnk (Bonfini *et al.* 1992; Therrien *et al.* 1995, 1998). In recent years, the fly eye has proven a powerful model for isolating specific regulators of oncogenic growth (*e.g.*, Xu *et al.* 1995; Moberg *et al.* 2001; Tapon *et al.* 2002). In this report, we demonstrate the further utility of the fly eye as an *in vivo* tool for addressing specific cancer syndromes.

One of our goals was to test previous proposals that Ret^{MEN2B} differed in its signaling specificity from *Ret* or Ret^{MEN2A}, which could account for the phenotypic differences between MEN2A and MEN2B (Songyang *et al.* 1995). However, our screens failed to identify any $dRet^{MEN2B}$-specific modifiers, despite testing >1000 essential genes plus 65% of the genome through deficiencies. While it is possible that we did not recover $dRet^{MEN2B}$-specific modifiers because we were unlucky and/or we did not assay for recessive modifiers, our results do not support the hypothesis that MEN2B-specific mutations create a shift in Ret specificity. A more attractive hypothesis to explain the phenotypic differences between MEN2A and MEN2B patients is that the mutations associated with these diseases differentially alter the regulation of the Ret kinase domain rather than its specificity. MEN2B mutations are clustered near the activation loop, a portion of the kinase domain that adopts an inhibitory conformation in resting RTKs (Hubbard and Till 2000). Molecular modeling and biochemical studies show that MEN2B mutations disrupt the inhibitory function of the activation loop to render Ret kinase active as a monomer and relieve Ret from control by *trans*-autophosphorylation (Iwashita *et al.* 1999; Miller *et al.* 2001). While Ret^{MEN2B} can be activated in a dimerization-independent manner, Ret^{MEN2A} protein is activated by constitutive dimerization and should be subject to regulators of the extracellular domain. Surprisingly, we isolated mutations that differentially modified $dRet^{MEN2A}$, perhaps by affecting constitutive dimerization. In support of this view, data from mouse models suggest that GFRα proteins interfere with Ret^{MEN2A} constitutive dimerization and possibly alter Ret^{MEN2A}-transforming activity (Kawai *et al.* 2000).

Although we identified numerous $dRet^{MEN2}$ genetic modifiers, most fell within limited classes. This leads us to conclude that a few pathways are fundamental for mediating *GMR-dRet* activity. Our studies clearly demonstrate that the SOS/Ras/ERK pathway is upregulated in *GMR-dRet*^{MEN2} flies: for example, eye tissue from *GMR-dRet*^{MEN2B} flies showed increased levels of activated diphospo-ERK. In fact, *GMR-dRet*^{MEN2} phenotypes are very similar to phenotypes conferred by retinal-specific hyperactivation of the Ras pathway (Karim *et al.* 1996; Halfar *et al.* 2001; Reich and Shilo 2002). This is consistent with numerous studies demonstrating that activation of normal and oncogenic Ret leads to recruitment of the Shc, SNT/FRS2, and Grb2 adaptor proteins to mediate Ras activation and induction of ERK signaling (van Weering *et al.* 1995; Ohiwa *et al.* 1997; Chiariello *et al.* 1998; Califano *et al.* 2000; Hayashi *et al.* 2000; Kurokawa *et al.* 2001; Melillo *et al.* 2001). Similar results have also been observed in mouse models for MEN2 (Sweetser *et al.* 1999). These data all point to Ras signal transduction as the principal effector pathway of Ret^{MEN2}. Our screening of *P*-element collections and candidate genes indicated that the Src and Jun kinase pathways also play a prominent role in *GMR-dRet* activity.

Human orthologs of *GMR-dRet*^{MEN2} modifiers may have important roles in Ret^{MEN2}-mediated tumorigenesis. The human orthologs of suppressor genes, including members of the Ras, Src, and Jun kinase pathways, are excellent candidate drug targets for rational drug design: in the fly eye, decreasing the genetic dose of these modifiers by half was often sufficient to dramatically reduce the effects of Ret^{MEN2} oncogenic activity *in situ*. The human orthologs of enhancer genes are also of interest because they may encode potential tumor suppressors or susceptibility loci. For example, human orthologs of dRet enhancer genes could be candidate modifiers in human MEN2 diseases. Perhaps individuals with germline mutations or allelic variants in these second-site modifiers are predisposed to more aggressive MTC or to the development of pheochromocytoma.

In human cancers, loss-of-function mutations in tumor suppressors cooperate with, or enhance, gain-of-function mutations in oncogenes such as Ras to promote tumorigenesis. To explore the role of putative tumor

suppressors for MTC and pheochromoctyoma, we examined the relationship between Ret^{MEN2} and human orthologs of *GMR-dRet* enhancers through direct study of primary tumor specimens. Tumor specimens from both sporadic and MEN2-associated MTCs and pheochromocytomas were examined for mutations in at least one allele of certain "enhancer" loci by scanning for somatic deletions or LOH. No MTCs showed consistent LOH, but pheochromocytomas showed LOH of two loci.

The *TNIK* locus showed LOH in six of seven informative pheochromocytoma cases. *TNIK*, an *msn* ortholog, maps to the long arm of human chromosome 3 (3q). While further analysis with flanking markers showed that these tumors have large deletions of 3q, no tumor suppressors that would clearly account for the selective loss of this region in pheochromocytomas map to 3q, leaving *TNIK* a viable candidate tumor suppressor. How TNIK could act as a tumor suppressor is undetermined. In mammalian tissue culture cells, TNIK protein binds to the Traf2 and Nck adaptor proteins, activates Jun kinase signaling, and inhibits cell adhesion (Fu *et al.* 1999). Perhaps *TNIK*, like Drosophila *msn*, negatively regulates Ras pathway signaling.

Pheochromocytomas also showed LOH of the candidate tumor suppressor *CHD3*. *CHD3*, also called *Mi-2*α, encodes a functional ortholog of *dMi-2* that is a core component of the NuRD chromatin-remodeling complex (Tong *et al.* 1998; Xue *et al.* 1998). Other NuRD components, such as MTA1 and MTA3, may play a role in metastatic breast cancer (Toh *et al.* 1995; Fujita *et al.* 2003). Perhaps *CHD3* mutations potentiate Ras signaling in human tissues just as mutations in Mi-2 orthologs enhanced the effects of Ras signaling in Drosophila and *C. elegans*. *CHD3* maps to 17p, a chromosomal arm that shows large deletions in pheochromocytomas (Khosla *et al.* 1991; Mulligan *et al.* 1993a; our data). A known tumor suppressor, *p53*, maps to this region, although previous analyses of 17p LOH in MEN2-associated pheochromocytomas failed to find any mutations in the remaining *p53* allele (Herfarth *et al.* 1997).

Pheochromocytoma occurs in a subset of MEN2 patients and typically shows far more chromosomal abnormalities than MTC, indicating that secondary genetic changes likely promote initiation or progression of this tumor type. Loss of *TNIK* and/or *CHD3* could be involved in this process. In a preliminary attempt to further characterize the role of these loci in pheochromocytoma, we searched for obvious mutations in the remaining *TNIK* and *CHD3* alleles in primary tumor specimens because, for classical tumor suppressors, mutation of both alleles must occur to provoke oncogenesis. These sequencing data confirmed the *TNIK* and *CHD3* LOH, but the remaining *TNIK* and *CHD3* alleles in those cases with LOH did not show any clear mutations, nor were mutations identified in tumors lacking LOH. However, in our Drosophila model, both *msn* and *dMi-2* enhance *GMR-dRet^{MEN2}* when just one functional copy of either gene is removed; the same could be true in human adrenal tissue. Perhaps loss of one allele, or haplo-insufficiency, of *TNIK* and/or *CHD3* cooperates with Ret^{MEN2} within adrenal tissues to promote tumorigenesis. In recent years, it has become clear that haplo-insufficiency at numerous tumor-suppressor genes, even classical tumor-suppressor genes, contributes to tumorigenesis in a variety of cancers (reviewed in Santarosa and Ashworth 2004). For example, loss of a single allele of *PTEN*, a classical tumor suppressor, is sufficient to promote prostate tumors, especially in the presence of a transforming oncogene (Di Cristofano *et al.* 1998; Kwabi-Addo *et al.* 2001). In the human adrenal gland, haplo-insufficiency at a tumor-suppressor locus may itself be sufficient to contribute to tumorigenesis in the context of a dominant transforming oncogene such as Ret^{MEN2}. For these reasons, assessing the potential of other members of our set of genetic modifiers to act as second-site modifiers in MEN2 tumors may prove useful.

We thank Brian Buntaine, who aided in fly care and injections, inverse PCR, and sequencing of *P*-element lines, and Chris Muriel, who aided with injections. We also thank Susan Spencer for guidance and discussion of unpublished data. We thank the Washington University Genome Sequencing Center for help with sequence analyses of *TNIK* and *CHD3*. We also thank Andrew McCallion and Arvinda Chakravarti for discussion of unpublished data regarding modifiers of human *c-ret*. This research was supported by a grant from the National Cancer Institute (5R01CA084309) to R. L. Cagan and by a National Institutes of Health training grant to R. D. Read.

LITERATURE CITED

Acton, D. S., D. Velthuyzen, C. J. Lips and J. W. Hoppener, 2000 Multiple endocrine neoplasia type 2B mutation in human RET oncogene induces medullary thyroid carcinoma in transgenic mice. Oncogene **19:** 3121–3125.

Ahringer, J., 2000 NuRD and SIN3 histone deacetylase complexes in development. Trends Genet. **16:** 351–356.

Asai, N., T. Iwashita, M. Matsuyama and M. Takahashi, 1995 Mechanism of activation of the ret proto-oncogene by multiple endocrine neoplasia 2A mutations. Mol. Cell. Biol. **15:** 1613–1619.

Asha, H., I. Nagy, G. Kovacs, D. Stetson, I. Ando *et al.*, 2003 Analysis of Ras-induced overproliferation in Drosophila hemocytes. Genetics **163:** 203–215.

Baker, N. E., and G. M. Rubin, 1992 Ellipse mutations in the Drosophila homologue of the EGF receptor affect pattern formation, cell division, and cell death in eye imaginal discs. Dev. Biol. **150:** 381–396.

Baker, N. E., and S. Y. Yu, 2001 The EGF receptor defines domains of cell cycle progression and survival to regulate cell number in the developing Drosophila eye. Cell **104:** 699–708.

Baloh, R. H., M. G. Tansey, P. A. Lampe, T. J. Fahrner, H. Enomoto *et al.*, 1998 Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron **21:** 1291–1302.

Besset, V., R. P. Scott and C. F. Ibanez, 2000 Signaling complexes and protein-protein interactions involved in the activation of the Ras and phosphatidylinositol 3-kinase pathways by the c-Ret receptor tyrosine kinase. J. Biol. Chem. **275:** 39159–39166.

Biggs, W. H., III, K. H. Zavitz, B. Dickson, A. van der Straten, D. Brunner *et al.*, 1994 The Drosophila rolled locus encodes a MAP kinase required in the sevenless signal transduction pathway. EMBO J. **13:** 1628–1635.

Bishop, J. G., III, and V. G. Corces, 1988 Expression of an activated ras gene causes developmental abnormalities in transgenic Drosophila melanogaster. Genes Dev. **2:** 567–577.

Bjorge, J. D., A. Jakymiw and D. J. Fujita, 2000 Selected glimpses into the activation and function of Src kinase. Oncogene **19:** 5620–5635.

Blume-Jensen, P., and T. Hunter, 2001 Oncogenic kinase signalling. Nature **411:** 355–365.

Bonfini, L., C. A. Karlovich, C. Dasgupta and U. Banerjee, 1992 The Son of sevenless gene product: a putative activator of Ras. Science **255:** 603–606.

Bongarzone, I., M. G. Butti, S. Coronelli, M. G. Borrello, M. Santoro *et al.*, 1994 Frequent activation of ret protooncogene by fusion with a new activating gene in papillary thyroid carcinomas. Cancer Res. **54:** 2979–2985.

Borrello, M. G., L. Alberti, E. Arighi, I. Bongarzone, C. Battistini *et al.*, 1996 The full oncogenic activity of Ret/ptc2 depends on tyrosine 539, a docking site for phospholipase Cgamma. Mol. Cell. Biol. **16:** 2151–2163.

Boutros, M., N. Paricio, D. I. Strutt and M. Mlodzik, 1998 Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. Cell **94:** 109–118.

Brandi, M. L., R. F. Gagel, A. Angeli, J. P. Bilezikian, P. Beck-Peccoz *et al.*, 2001 Guidelines for diagnosis and therapy of MEN type 1 and type 2. J. Clin. Endocrinol. Metab. **86:** 5658–5671.

Brehm, A., G. Langst, J. Kehle, C. R. Clapier, A. Imhof *et al.*, 2000 dMi-2 and ISWI chromatin remodelling factors have distinct nucleosome binding and mobilization properties. EMBO J. **19:** 4332–4341.

Brown, N. H., 1994 Null mutations in the alpha PS2 and beta PS integrin subunit genes have distinct phenotypes. Development **120:** 1221–1231.

Buj-Bello, A., J. Adu, L. G. Pinon, A. Horton, J. Thompson *et al.*, 1997 Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature **387:** 721–724.

Califano, D., C. Rizzo, A. D'Alessio, G. L. Colucci-D'Amato, G. Cali *et al.*, 2000 Signaling through Ras is essential for ret oncogene-induced cell differentiation in PC12 cells. J. Biol. Chem. **275:** 19297–19305.

Carlson, K. M., J. Bracamontes, C. E. Jackson, R. Clark, A. Lacroix *et al.*, 1994a Parent-of-origin effects in multiple endocrine neoplasia type 2B. Am. J. Hum. Genet. **55:** 1076–1082.

Carlson, K. M., S. Dou, D. Chi, N. Scavarda, K. Toshima *et al.*, 1994b Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B. Proc. Natl. Acad. Sci. USA **91:** 1579–1583.

Carmena, A., E. Buff, M. S. Halfon, S. Gisselbrecht, F. Jimenez *et al.*, 2002 Reciprocal regulatory interactions between the Notch and Ras signaling pathways in the Drosophila embryonic mesoderm. Dev. Biol. **244:** 226–242.

Charrasse, S., M. Meriane, F. Comunale, A. Blangy and C. Gauthier-Rouviere, 2002 N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. J. Cell Biol. **158:** 953–965.

Chellaiah, M., N. Kizer, M. Silva, U. Alvarez, D. Kwiatkowski *et al.*, 2000 Gelsolin deficiency blocks podosome assembly and produces increased bone mass and strength. J. Cell Biol. **148:** 665–678.

Chen, Z., and M. Han, 2001 C. elegans Rb, NuRD, and Ras regulate lin-39-mediated cell fusion during vulval fate specification. Curr. Biol. **11:** 1874–1879.

Chiariello, M., R. Visconti, F. Carlomagno, R. M. Melillo, C. Bucci *et al.*, 1998 Signalling of the Ret receptor tyrosine kinase through the c-Jun NH2-terminal protein kinases (JNKS): evidence for a divergence of the ERKs and JNKs pathways induced by Ret. Oncogene **16:** 2435–2445.

Chiefari, E., D. Russo, D. Giuffrida, G. A. Zampa, D. Meringolo *et al.*, 1998 Analysis of RET proto-oncogene abnormalities in patients with MEN 2A, MEN 2B, familial or sporadic medullary thyroid carcinoma. J. Endocrinol. Invest. **21:** 358–364.

Cranston, A. N., and B. A. J. Ponder, 2003 Modulation of medullary thyroid carcinoma penetrance suggests the presence of modifier genes in a RET transgenic mouse model. Cancer Res. **63:** 4777–4780.

Cullis, D. N., B. Philip, J. D. Baleja and L. A. Feig, 2002 Rab11-FIP2, an adaptor protein connecting cellular components involved in internalization and recycling of epidermal growth factor receptors. J. Biol. Chem. **277:** 49158–49166.

Deak, P., M. M. Omar, R. D. Saunders, M. Pal, O. Komonyi *et al.*, 1997 *P*-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: correlation of physical and cytogenetic maps in chromosomal region 86E–87F. Genetics **147:** 1697–1722.

Dickson, B., F. Sprenger, D. Morrison and E. Hafen, 1992 Raf functions downstream of Ras1 in the Sevenless signal transduction pathway. Nature **360:** 600–603.

Di Cristofano, A., B. Pesce, C. Cordon-Cardo and P. P. Pandolfi, 1998 Pten is essential for embryonic development and tumour suppression. Nat. Genet. **19:** 348–355.

Dodson, G. S., D. J. Guarnieri and M. A. Simon, 1998 Src64 is required for ovarian ring canal morphogenesis during Drosophila oogenesis. Development **125:** 2883–2892.

Dong, X., L. Tsuda, K. H. Zavitz, M. Lin, S. Li *et al.*, 1999 ebi regulates epidermal growth factor receptor signaling pathways in Drosophila. Genes Dev. **13:** 954–965.

Donis-Keller, H., S. Dou, D. Chi, K. M. Carlson, K. Toshima *et al.*, 1993 Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. Hum. Mol. Genet. **2:** 851–856.

Durbec, P., C. V. Marcos-Gutierrez, C. Kilkenny, M. Grigoriou, K. Wartiowaara *et al.*, 1996 GDNF signalling through the Ret receptor tyrosine kinase. Nature **381:** 789–793.

Easton, D. F., M. A. Ponder, T. Cummings, R. F. Gagel, H. H. Hansen *et al.*, 1989 The clinical and screening age-at-onset distribution for the MEN-2 syndrome. Am. J. Hum. Genet. **44:** 208–215.

Encinas, M., M. G. Tansey, B. A. Tsui-Pierchala, J. X. Comella, J. Milbrandt *et al.*, 2001 c-Src is required for glial cell line-derived neurotrophic factor (GDNF) family ligand-mediated neuronal survival via a phosphatidylinositol-3 kinase (PI-3K)-dependent pathway. J. Neurosci. **21:** 1464–1472.

Eng, C., D. P. Smith, L. M. Mulligan, M. A. Nagai, C. S. Healey *et al.*, 1994 Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. Hum. Mol. Genet. **3:** 237–241.

Eng, C., D. Clayton, I. Schuffenecker, G. Lenoir, G. Cote *et al.*, 1996 The relationship between specific RET proto-oncogene mutations and disease phenotype in multiple endocrine neoplasia type 2. International RET mutation consortium analysis. JAMA **276:** 1575–1579.

Enokido, Y., F. de Sauvage, J. A. Hongo, N. Ninkina, A. Rosenthal *et al.*, 1998 GFR alpha-4 and the tyrosine kinase Ret form a functional receptor complex for persephin. Curr. Biol. **8:** 1019–1022.

Enomoto, H., T. Araki, A. Jackman, R. O. Heuckeroth, W. D. Snider *et al.*, 1998 GFR alpha1-deficient mice have deficits in the enteric nervous system and kidneys. Neuron **21:** 317–324.

Enomoto, H., P. A. Crawford, A. Gorodinsky, R. O. Heuckeroth, E. M. Johnson, Jr. *et al.*, 2001 RET signaling is essential for migration, axonal growth and axon guidance of developing sympathetic neurons. Development **128:** 3963–3974.

Fortini, M. E., M. A. Simon and G. M. Rubin, 1992 Signalling by the sevenless protein tyrosine kinase is mimicked by Ras1 activation. Nature **355:** 559–561.

Freeman, M., 1996 Reiterative use of the EGF receptor triggers differentiation of all cell types in the Drosophila eye. Cell **87:** 651–660.

Fu, C. A., M. Shen, B. C. Huang, J. Lasaga, D. G. Payan *et al.*, 1999 TNIK, a novel member of the germinal center kinase family that activates the c-Jun N-terminal kinase pathway and regulates the cytoskeleton. J. Biol. Chem. **274:** 30729–30737.

Fujita, N., D. L. Jaye, M. Kajita, C. Geigerman, C. S. Moreno *et al.*, 2003 MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell **113:** 207–219.

Garrity, P. A., Y. Rao, I. Salecker, J. McGlade, T. Pawson *et al.*, 1996 Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. Cell **85:** 639–650.

Gordon, D., C. Abajian and P. Green, 1998 Consed: a graphical tool for sequence finishing. Genome Res. **8:** 195–202.

Grieco, M., M. Santoro, M. T. Berlingieri, R. M. Melillo, R. Donghi *et al.*, 1990 PTC is a novel rearranged form of the

ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell **60:** 557–563.

Hahn, M., and J. Bishop, 2001 Expression pattern of Drosophila ret suggests a common ancestral origin between the metamorphosis precursors in insect endoderm and the vertebrate enteric neurons. Proc. Natl. Acad. Sci. USA **98:** 1053–1058.

Halfar, K., C. Rommel, H. Stocker and E. Hafen, 2001 Ras controls growth, survival and differentiation in the Drosophila eye by different thresholds of MAP kinase activity. Development **128:** 1687–1696.

Hashino, E., M. Shero, D. Junghans, H. Rohrer, J. Milbrandt *et al.*, 2001 GDNF and neurturin are target-derived factors essential for cranial parasympathetic neuron development. Development **128:** 3773–3782.

Hayashi, H., M. Ichihara, T. Iwashita, H. Murakami, Y. Shimono *et al.*, 2000 Characterization of intracellular signals via tyrosine 1062 in RET activated by glial cell line-derived neurotrophic factor. Oncogene **19:** 4469–4475.

Heberlein, U., C. M. Singh, A. Y. Luk and T. J. Donohoe, 1995 Growth and differentiation in the Drosophila eye coordinated by hedgehog. Nature **373:** 709–711.

Herfarth, K. K., M. R. Wick, H. N. Marshall, E. Gartner, S. Lum *et al.*, 1997 Absence of TP53 alterations in pheochromocytomas and medullary thyroid carcinomas. Genes Chromosomes Cancer **20:** 24–29.

Heuckeroth, R. O., P. A. Lampe, E. M. Johnson and J. Milbrandt, 1998 Neurturin and GDNF promote proliferation and survival of enteric neuron and glial progenitors in vitro. Dev. Biol. **200:** 116–129.

Hofstra, R. M., R. M. Landsvater, I. Ceccherini, R. P. Stulp, T. Stelwagen *et al.*, 1994 A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature **367:** 375–376.

Huang, A. M., and G. M. Rubin, 2000 A misexpression screen identifies genes that can modulate RAS1 pathway signaling in *Drosophila melanogaster.* Genetics **156:** 1219–1230.

Hubbard, S. R., and J. H. Till, 2000 Protein tyrosine kinase structure and function. Annu. Rev. Biochem. **69:** 373–398.

Islam, R., S. Y. Wei, W. H. Chiu, M. Hortsch and J. C. Hsu, 2003 Neuroglian activates Echinoid to antagonize the Drosophila EGF receptor signaling pathway. Development **130:** 2051–2059.

Iwashita, T., N. Asai, H. Murakami, M. Matsuyama and M. Takahashi, 1996 Identification of tyrosine residues that are essential for transforming activity of the ret proto-oncogene with MEN2A or MEN2B mutation. Oncogene **12:** 481–487.

Iwashita, T., M. Kato, H. Murakami, N. Asai, Y. Ishiguro *et al.*, 1999 Biological and biochemical properties of Ret with kinase domain mutations identified in multiple endocrine neoplasia type 2B and familial medullary thyroid carcinoma. Oncogene **18:** 3919–3922.

Jing, S., D. Wen, Y. Yu, P. L. Holst, Y. Luo *et al.*, 1996 GDNF-induced activation of the ret protein tyrosine kinase is mediated by GDNFR-alpha, a novel receptor for GDNF. Cell **85:** 1113–1124.

Karim, F. D., H. C. Chang, M. Therrien, D. A. Wassarman, T. Laverty *et al.*, 1996 A screen for genes that function downstream of Ras1 during Drosophila eye development. Genetics **143:** 315–329.

Kawai, K., T. Iwashita, H. Murakami, N. Hiraiwa, A. Yoshiki *et al.*, 2000 Tissue-specific carcinogenesis in transgenic mice expressing the RET proto-oncogene with a multiple endocrine neoplasia type 2A mutation. Cancer Res. **60:** 5254–5260.

Kehle, J., D. Beuchle, S. Treuheit, B. Christen, J. A. Kennison *et al.*, 1998 dMi-2, a hunchback-interacting protein that functions in polycomb repression. Science **282:** 1897–1900.

Khosla, S., V. M. Patel, I. D. Hay, D. J. Schaid, C. S. Grant *et al.*, 1991 Loss of heterozygosity suggests multiple genetic alterations in pheochromocytomas and medullary thyroid carcinomas. J. Clin. Invest. **87:** 1691–1699.

Klein, R. D., D. Sherman, W. H. Ho, D. Stone, G. L. Bennett *et al.*, 1997 A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature **387:** 717–721.

Kotzbauer, P. T., P. A. Lampe, R. O. Heuckeroth, J. P. Golden, D. J. Creedon *et al.*, 1996 Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature **384:** 467–470.

Kurokawa, K., T. Iwashita, H. Murakami, H. Hayashi, K. Kawai *et al.*, 2001 Identification of SNT/FRS2 docking site on RET receptor tyrosine kinase and its role for signal transduction. Oncogene **20:** 1929–1938.

Kurokawa, K., K. Kawai, M. Hashimoto, Y. Ito and M. Takahashi, 2003 Cell signalling and gene expression mediated by RET tyrosine kinase. J. Intern. Med. **253:** 627–633.

Kusano, K., D. M. Johnson-Schlitz and W. R. Engels, 2001 Sterility of Drosophila with mutations in the Bloom syndrome gene—complementation by Ku70. Science **291:** 2600–2602.

Kwabi-Addo, B., D. Giri, K. Schmidt, K. Podsypanina, R. Parsons *et al.*, 2001 Haploinsufficiency of the Pten tumor suppressor gene promotes prostate cancer progression. Proc. Natl. Acad. Sci. USA **98:** 11563–11568.

Lai, Z. C., and G. M. Rubin, 1992 Negative control of photoreceptor development in Drosophila by the *product* of the yan gene, an ETS domain protein. Cell **70:** 609–620.

Lai, Z. C., S. D. Harrison, F. Karim, Y. Li and G. M. Rubin, 1996 Loss of tramtrack gene activity results in ectopic R7 cell formation, even in a sina mutant background. Proc. Natl. Acad. Sci. USA **93:** 5025–5030.

Leboulleux, S., E. Baudin, J. P. Travagli and M. Schlumberger, 2004 Medullary thyroid carcinoma. Clin. Endocrinol. **61:** 299–310.

Lee, J. R., S. Urban, C. F. Garvey and M. Freeman, 2001 Regulated intracellular ligand transport and proteolysis control EGF signal activation in Drosophila. Cell **107:** 161–171.

Lesokhin, A. M., S. Y. Yu, J. Katz and N. E. Baker, 1999 Several levels of EGF receptor signaling during photoreceptor specification in wild-type, Ellipse, and null mutant Drosophila. Dev. Biol. **205:** 129–144.

Ley, T. J., P. J. Minx, M. J. Walter, R. E. Ries, H. Sun *et al.*, 2003 A pilot study of high-throughput, sequence-based mutational profiling of primary human acute myeloid leukemia cell genomes. Proc. Natl. Acad. Sci. USA **100:** 14275–14280.

Liu, X., Q. C. Vega, R. A. Decker, A. Pandey, C. A. Worby *et al.*, 1996 Oncogenic RET receptors display different autophosphorylation sites and substrate binding specificities. J. Biol. Chem. **271:** 5309–5312.

Maixner, A., T. P. Hecker, Q. N. Phan and D. A. Wassarman, 1998 A screen for mutations that prevent lethality caused by expression of activated sevenless and Ras1 in the Drosophila embryo. Dev. Genet. **23:** 347–361.

Marsh, D. J., G. Theodosopoulos, K. Martin-Schulte, A. L. Richardson, J. Philips *et al.*, 2003 Genome-wide copy number imbalances identified in familial and sporadic medullary thyroid carcinoma. J. Clin. Endocrinol. Metab. **88:** 1866–1872.

Marshall, G. M., A. E. Peaston, J. E. Hocker, S. A. Smith, L. M. Hansford *et al.*, 1997 Expression of multiple endocrine neoplasia 2B RET in neuroblastoma cells alters cell adhesion in vitro, enhances metastatic behavior in vivo, and activates Jun kinase. Cancer Res. **57:** 5399–5405.

Matakatsu, H., R. Tadokoro, S. Gamo and S. Hayashi, 1999 Repression of the wing vein development in Drosophila by the nuclear matrix protein plexus. Development **126:** 5207–5216.

Matias-Guiu, X., G. Peiro, J. Esquius, E. Oliva, R. Cabezas *et al.*, 1995 Proliferative activity in C-cell hyperplasia and medullary thyroid carcinoma. Evaluation by PCNA immunohistochemistry and AgNORs staining. Pathol. Res. Pract. **191:** 42–47.

Melillo, R. M., M. V. Barone, G. Lupoli, A. M. Cirafici, F. Carlomagno *et al.*, 1999 Ret-mediated mitogenesis requires Src kinase activity. Cancer Res. **59:** 1120–1126.

Melillo, R. M., M. Santoro, S. H. Ong, M. Billaud, A. Fusco *et al.*, 2001 Docking protein FRS2 links the protein tyrosine kinase RET and its oncogenic forms with the mitogen-activated protein kinase signaling cascade. Mol. Cell. Biol. **21:** 4177–4187.

Menko, F. H., R. B. van der Luijt, I. A. de Valk, A. W. Toorians, J. M. Sepers *et al.*, 2002 Atypical MEN type 2B associated with two germline RET mutations on the same allele not involving codon 918. J. Clin. Endocrinol. Metab. **87:** 393–397.

Michiels, F. M., S. Chappuis, B. Caillou, A. Pasini, M. Talbot *et al.*, 1997 Development of medullary thyroid carcinoma in transgenic mice expressing the RET protooncogene altered by a multiple endocrine neoplasia type 2A mutation. Proc. Natl. Acad. Sci. USA **94:** 3330–3335.

Milbrandt, J., F. J. de Sauvage, T. J. Fahrner, R. H. Baloh, M. L. Leitner *et al.*, 1998 Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron **20:** 245–253.

Miller, D. T., and R. L. Cagan, 1998 Local induction of patterning and programmed cell death in the developing Drosophila retina. Development **125:** 2327–2335.

Miller, M., K. Ginalski, B. Lesyng, N. Nakaigawa, L. Schmidt *et al.*, 2001 Structural basis of oncogenic activation caused by point mutations in the kinase domain of the MET proto-oncogene: modeling studies. Proteins **44:** 32–43.

Miyauchi, A., H. Futami, N. Hai, T. Yokozawa, K. Kuma *et al.*, 1999 Two germline missense mutations at codons 804 and 806 of the RET proto-oncogene in the same allele in a patient with multiple endocrine neoplasia type 2B without codon 918 mutation. Jpn. J. Cancer Res. **90:** 1–5.

Moberg, K. H., D. W. Bell, D. C. Wahrer, D. A. Haber and I. K. Hariharan, 2001 Archipelago regulates cyclin E levels in Drosophila and is mutated in human cancer cell lines. Nature **413:** 311–316.

Mulligan, L. M., E. Gardner, B. A. Smith, C. G. Mathew and B. A. Ponder, 1993a Genetic events in tumour initiation and progression in multiple endocrine neoplasia type 2. Genes Chromosomes Cancer **6:** 166–177.

Mulligan, L. M., J. B. Kwok, C. S. Healey, M. J. Elsdon, C. Eng *et al.*, 1993b Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature **363:** 458–460.

Mulligan, L. M., C. Eng, C. S. Healey, D. Clayton, J. B. Kwok *et al.*, 1994a Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. Nat. Genet. **6:** 70–74.

Mulligan, L. M., C. Eng, C. S. Healey, M. A. Ponder, G. L. Feldman *et al.*, 1994b A de novo mutation of the RET proto-oncogene in a patient with MEN 2A. Hum. Mol. Genet. **3:** 1007–1008.

Murakami, H., T. Iwashita, N. Asai, Y. Iwata, S. Narumiya *et al.*, 1999a Rho-dependent and -independent tyrosine phosphorylation of focal adhesion kinase, paxillin and p130Cas mediated by Ret kinase. Oncogene **18:** 1975–1982.

Murakami, H., T. Iwashita, N. Asai, Y. Shimono, Y. Iwata *et al.*, 1999b Enhanced phosphatidylinositol 3-kinase activity and high phosphorylation state of its downstream signalling molecules mediated by ret with the MEN 2B mutation. Biochem. Biophys. Res. Commun. **262:** 68–75.

Murawsky, C. M., A. Brehm, P. Badenhorst, N. Lowe, P. B. Becker *et al.*, 2001 Tramtrack69 interacts with the dMi-2 subunit of the Drosophila NuRD chromatin remodelling complex. EMBO Rep. **2:** 1089–1094.

Natarajan, D., C. Marcos-Gutierrez, V. Pachnis and E. de Graaff, 2002 Requirement of signalling by receptor tyrosine kinase RET for the directed migration of enteric nervous system progenitor cells during mammalian embryogenesis. Development **129:** 5151–5160.

Neufeld, T. P., A. H. Tang and G. M. Rubin, 1998 A genetic screen to identify components of the sina signaling pathway in Drosophila eye development. Genetics **148:** 277–286.

Nickerson, D. A., V. O. Tobe and S. L. Taylor, 1997 PolyPhred: automating the detection and genotyping of single nucleotide substitutions using fluorescence-based resequencing. Nucleic Acids Res. **25:** 2745–2751.

Ohiwa, M., H. Murakami, T. Iwashita, N. Asai, Y. Iwata *et al.*, 1997 Characterization of Ret-Shc-Grb2 complex induced by GDNF, MEN 2A, and MEN 2B mutations. Biochem. Biophys. Res. Commun. **237:** 747–751.

Okamura, H., and M. D. Resh, 1995 p80/85 cortactin associates with the Src SH2 domain and colocalizes with v-Src in transformed cells. J. Biol. Chem. **270:** 26613–26618.

O'Riordain, D. S., T. O'Brien, T. B. Crotty, H. Gharib, C. S. Grant *et al.*, 1995 Multiple endocrine neoplasia type 2B: more than an endocrine disorder. Surgery **118:** 936–942.

Paricio, N., F. Feiguin, M. Boutros, S. Eaton and M. Mlodzik, 1999 The Drosophila STE20-like kinase misshapen is required downstream of the Frizzled receptor in planar polarity signaling. EMBO J. **18:** 4669–4678.

Peiffer, S. L., T. J. Herzog, D. J. Tribune, D. G. Mutch, D. J. Gersell *et al.*, 1995 Allelic loss of sequences from the long arm of chromosome 10 and replication errors in endometrial cancers. Cancer Res. **55:** 1922–1926.

Pennetta, G., and D. Pauli, 1998 The Drosophila Sin3 gene encodes a widely distributed transcription factor essential for embryonic viability. Dev. Genes Evol. **208:** 531–536.

Ponder, B. A., 1999 The phenotypes associated with ret mutations in the multiple endocrine neoplasia type 2 syndrome. Cancer Res. **59:** 1736s–1741s; discussion 1742s.

Prokopenko, S. N., A. Brumby, L. O'Keefe, L. Prior, Y. He *et al.*, 1999 A putative exchange factor for Rho1 GTPase is required for initiation of cytokinesis in Drosophila. Genes Dev. **13:** 2301–2314.

Quayle, F. J., and J. F. Moley, 2005 Medullary thyroid carcinoma: including MEN 2A and MEN 2B syndromes. J. Surg. Oncol. **89:** 122–129.

Read, R. D., E. A. Bach and R. L. Cagan, 2004 Drosophila C-terminal Src kinase negatively regulates organ growth and cell proliferation through inhibition of the Src, Jun N-terminal kinase, and STAT pathways. Mol. Cell. Biol. **24:** 6676–6689.

Rebay, I., F. Chen, F. Hsiao, P. A. Kolodziej, B. H. Kuang *et al.*, 2000 A genetic screen for novel components of the Ras/mitogen-activated protein kinase signaling pathway that interact with the *yan* gene of Drosophila identifies split ends, a new RNA recognition motif-containing protein. Genetics **154:** 695–712.

Reich, A., and B. Z. Shilo, 2002 Keren, a new ligand of the Drosophila epidermal growth factor receptor, undergoes two modes of cleavage. EMBO J. **21:** 4287–4296.

Ruan, W., P. Pang and Y. Rao, 1999 The SH2/SH3 adaptor protein dock interacts with the Ste20-like kinase misshapen in controlling growth cone motility. Neuron **24:** 595–605.

Ruan, W., H. Long, D. H. Vuong and Y. Rao, 2002 Bifocal is a downstream target of the Ste20-like serine/threonine kinase misshapen in regulating photoreceptor growth cone targeting in Drosophila. Neuron **36:** 831–842.

Salvatore, D., R. M. Melillo, C. Monaco, R. Visconti, G. Fenzi *et al.*, 2001 Increased in vivo phosphorylation of ret tyrosine 1062 is a potential pathogenetic mechanism of multiple endocrine neoplasia type 2B. Cancer Res. **61:** 1426–1431.

Salzberg, A., S. N. Prokopenko, Y. He, P. Tsai, M. Pal *et al.*, 1997 *P*-element insertion alleles of essential genes on the third chromosome of *Drosophila melanogaster*: mutations affecting embryonic PNS development. Genetics **147:** 1723–1741.

Sanchez, M. P., I. Silos-Santiago, J. Frisen, B. He, S. A. Lira *et al.*, 1996 Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature **382:** 70–73.

Santarosa, M., and A. Ashworth, 2004 Haploinsufficiency for tumour suppressor genes: when you don't need to go all the way. Biochim. Biophys. Acta **1654:** 105–122.

Santoro, M., F. Carlomagno, A. Romano, D. P. Bottaro, N. A. Dathan *et al.*, 1995 Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science **267:** 381–383.

Sawamoto, K., A. Taguchi, Y. Hirota, C. Yamada, M. H. Jin *et al.*, 1998 Argos induces programmed cell death in the developing Drosophila eye by inhibition of the Ras pathway. Cell Death Differ. **5:** 262–270.

Schuchardt, A., V. D'Agati, L. Larsson-Blomberg, F. Costantini and V. Pachnis, 1994 Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature **367:** 380–383.

Scurini, C., L. Quadro, O. Fattoruso, U. Verga, A. Libroia *et al.*, 1998 Germline and somatic mutations of the RET proto-oncogene in apparently sporadic medullary thyroid carcinomas. Mol. Cell. Endocrinol. **137:** 51–57.

Segouffin-Cariou, C., and M. Billaud, 2000 Transforming ability of MEN2A-RET requires activation of the phosphatidylinositol 3-kinase/AKT signaling pathway. J. Biol. Chem. **275:** 3568–3576.

Shirahama, S., K. Ogura, H. Takami, K. Ito, T. Tohsen *et al.*, 1998 Mutational analysis of the RET proto-oncogene in 71 Japanese patients with medullary thyroid carcinoma. J. Hum. Genet. **43:** 101–106.

Simon, M. A., 2000 Receptor tyrosine kinases: specific outcomes from general signals. Cell **103:** 13–15.

Simon, M. A., D. D. Bowtell, G. S. Dodson, T. R. Laverty and G. M. Rubin, 1991 Ras1 and a putative guanine nucleotide exchange factor perform crucial steps in signaling by the sevenless protein tyrosine kinase. Cell **67:** 701–716.

Smith, D. P., C. Houghton and B. A. Ponder, 1997 Germline mutation of RET codon 883 in two cases of de novo MEN 2B. Oncogene **15:** 1213–1217.

Smith-Hicks, C. L., K. C. Sizer, J. F. Powers, A. S. Tischler and F. Costantini, 2000 C-cell hyperplasia, pheochromocytoma and sympathoadrenal malformation in a mouse model of multiple endocrine neoplasia type 2B. EMBO J. **19:** 612–622.

Solari, F., A. Bateman and J. Ahringer, 1999 The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development **126:** 2483–2494.

Soler, R. M., X. Dolcet, M. Encinas, J. Egea, J. R. Bayascas *et al.*, 1999 Receptors of the glial cell line-derived neurotrophic factor family of neurotrophic factors signal cell survival through the phosphatidylinositol 3-kinase pathway in spinal cord motoneurons. J. Neurosci. **19:** 9160–9169.

Songyang, Z., K. Carraway, M. Eck, S. Harrison, R. Feldman *et al.*, 1995 Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature **373:** 536–539.

Speck, O., S. C. Hughes, N. K. Noren, R. M. Kulikauskas and R. G. Fehon, 2003 Moesin functions antagonistically to the Rho pathway to maintain epithelial integrity. Nature **421:** 83–87.

Spencer, S. A., and R. L. Cagan, 2003 Echinoid is essential for regulation of Egfr signaling and R8 formation during Drosophila eye development. Development **130:** 3725–3733.

Spencer, S. A., P. A. Powell, D. T. Miller and R. L. Cagan, 1998 Regulation of EGF receptor signaling establishes pattern across the developing Drosophila retina. Development **125:** 4777–4790.

Spradling, A. C., D. Stern, A. Beaton, E. J. Rhem, T. Laverty *et al.*, 1999 The Berkeley *Drosophila* Genome Project gene disruption project: single *P*-element insertions mutating 25% of vital Drosophila genes. Genetics **153:** 135–177.

Stewart, R. A., D.-M. Li, H. Huang and T. Xu, 2003 A genetic screen for modifiers of the lats tumor suppressor gene identifies C-terminal Src kinase as a regulator of cell proliferation in Drosophila. Oncogene **22:** 6436–6444.

Stokes, D. G., K. D. Tartof and R. P. Perry, 1996 CHD1 is concentrated in interbands and puffed regions of Drosophila polytene chromosomes. Proc. Natl. Acad. Sci. USA **93:** 7137–7142.

Su, Y. C., J. E. Treisman and E. Y. Skolnik, 1998 The Drosophila Ste20-related kinase misshapen is required for embryonic dorsal closure and acts through a JNK MAPK module on an evolutionarily conserved signaling pathway. Genes Dev. **12:** 2371–2380.

Sugaya, R., S. Ishimaru, T. Hosoya, K. Saigo and Y. Emori, 1994 A Drosophila homolog of human proto-oncogene ret transiently expressed in embryonic neuronal precursor cells including neuroblasts and CNS cells. Mech. Dev. **45:** 139–145.

Sweetser, D. A., G. J. Froelick, A. M. Matsumoto, K. E. Kafer, B. Marck *et al.*, 1999 Ganglioneuromas and renal anomalies are induced by activated RET(MEN2B) in transgenic mice. Oncogene **18:** 877–886.

Takahashi, F., S. Endo, T. Kojima and K. Saigo, 1996 Regulation of cell-cell contacts in developing Drosophila eyes by Dsrc41, a new, close relative of vertebrate c-src. Genes Dev. **10:** 1645–1656.

Takahashi, M., 2001 The GDNF/RET signaling pathway and human diseases. Cytokine Growth Factor Rev. **12:** 361–373.

Tang, M. J., Y. Cai, S. J. Tsai, Y. K. Wang and G. R. Dressler, 2002 Ureteric bud outgrowth in response to RET activation is mediated by phosphatidylinositol 3-kinase. Dev. Biol. **243:** 128–136.

Tapon, N., K. F. Harvey, D. W. Bell, D. C. Wahrer, T. A. Schiripo *et al.*, 2002 salvador promotes both cell cycle exit and apoptosis in Drosophila and is mutated in human cancer cell lines. Cell **110:** 467–478.

Taraviras, S., C. V. Marcos-Gutierrez, P. Durbec, H. Jani, M. Grigoriou *et al.*, 1999 Signalling by the RET receptor tyrosine kinase and its role in the development of the mammalian enteric nervous system. Development **126:** 2785–2797.

Tautz, D., and C. Pfeifle, 1989 A non-radioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals translational control of the segmentation gene hunchback. Chromosoma **98:** 81–85.

Therrien, M., H. C. Chang, N. M. Solomon, F. D. Karim, D. A. Wassarman *et al.*, 1995 KSR, a novel protein kinase required for RAS signal transduction. Cell **83:** 879–888.

Therrien, M., A. M. Wong and G. M. Rubin, 1998 CNK, a RAF-binding multidomain protein required for RAS signaling. Cell **95:** 343–353.

Therrien, M., D. K. Morrison, A. M. Wong and G. M. Rubin, 2000 A genetic screen for modifiers of a kinase suppressor of Ras-dependent rough eye phenotype in Drosophila. Genetics **156:** 1231–1242.

Toh, Y., S. D. Pencil and G. L. Nicolson, 1995 Analysis of the complete sequence of the novel metastasis-associated candidate gene, mta1, differentially expressed in mammary adenocarcinoma and breast cancer cell lines. Gene **159:** 97–104.

Tong, J. K., C. A. Hassig, G. R. Schnitzler, R. E. Kingston and S. L. Schreiber, 1998 Chromatin deacetylation by an ATP-dependent nucleosome remodelling complex. Nature **395:** 917–921.

Treisman, J. E., N. Ito and G. M. Rubin, 1997 misshapen encodes a protein kinase involved in cell shape control in Drosophila. Gene **186:** 119–125.

Tsuda, L., R. Nagaraj, S. L. Zipursky and U. Banerjee, 2002 An EGFR/Ebi/Sno pathway promotes delta expression by inactivating Su(H)/SMRTER repression during inductive notch signaling. Cell **110:** 625–637.

Urban, S., J. R. Lee and M. Freeman, 2002 A family of rhomboid intramembrane proteases activates all Drosophila membrane-tethered EGF ligands. EMBO J. **21:** 4277–4286.

van Heurn, L. W., C. Schaap, G. Sie, A. A. Haagen, W. J. Gerver *et al.*, 1999 Predictive DNA testing for multiple endocrine neoplasia 2: a therapeutic challenge of prophylactic thyroidectomy in very young children. J. Pediatr. Surg. **34:** 568–571.

Van Vactor, D. L., Jr., R. L. Cagan, H. Kramer and S. L. Zipursky, 1991 Induction in the developing compound eye of Drosophila: multiple mechanisms restrict R7 induction to a single retinal precursor cell. Cell **67:** 1145–1155.

van Weering, D. H., J. P. Medema, A. van Puijenbroek, B. M. Burgering, P. D. Baas *et al.*, 1995 Ret receptor tyrosine kinase activates extracellular signal-regulated kinase 2 in SK-N-MC cells. Oncogene **11:** 2207–2214.

Voas, M. G., and I. Rebay, 2004 Signal integration during development: insights from the Drosophila eye. Dev. Dyn. **229:** 162–175.

von Zelewsky, T., F. Palladino, K. Brunschwig, H. Tobler, A. Hajnal *et al.*, 2000 The C. elegans Mi-2 chromatin-remodelling proteins function in vulval cell fate determination. Development **127:** 5277–5284.

Wolfe, H. J., K. E. Melvin, S. J. Cervi-Skinner, A. A. Saadi, J. F. Juliar *et al.*, 1973 C-cell hyperplasia preceding medullary thyroid carcinoma. N. Engl. J. Med. **289:** 437–441.

Wu, H., A. B. Reynolds, S. B. Kanner, R. R. Vines and J. T. Parsons, 1991 Identification and characterization of a novel cytoskeleton-associated pp60src substrate. Mol. Cell. Biol. **11:** 5113–5124.

Xu, T., W. Wang, S. Zhang, R. A. Stewart and W. Yu, 1995 Identifying tumor suppressors in genetic mosaics: the Drosophila lats gene encodes a putative protein kinase. Development **121:** 1053–1063.

Xue, Y., J. Wong, G. T. Moreno, M. K. Young, J. Cote *et al.*, 1998 NURD, a novel complex with both ATP-dependent chromatin-remodeling and histone deacetylase activities. Mol. Cell **2:** 851–861.

Yamashita, Y., S. Kajigaya, K. Yoshida, S. Ueno, J. Ota *et al.*, 2001 Sak serine-threonine kinase acts as an effector of Tec tyrosine kinase. J. Biol. Chem. **276:** 39012–39020.

Communicating editor: K. Golic