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Neutralizing Aptamers from Whole-Cell SELEX Inhibit the RET Receptor Tyrosine Kinase

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Targeting large transmembrane molecules, including receptor tyrosine kinases, is a major pharmacological challenge. Specific oligonucleotide ligands (aptamers) can be generated for a variety of targets through the iterative evolution of a random pool of sequences (SELEX). Nuclease-resistant aptamers that recognize the human receptor tyrosine kinase RET were obtained using RET-expressing cells as targets in a modified SELEX procedure. Remarkably, one of these aptamers blocked RET-dependent intracellular signaling pathways by interfering with receptor dimerization when the latter was induced by the physiological ligand or by an activating mutation. This strategy is generally applicable to transmembrane receptors and opens the way to targeting other members of this class of proteins that are of major biomedical importance.

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Introduction

The identification of tumor-specific molecular markers is a powerful tool in cancer diagnostics, and the targeting of tumor-specific pathways is the best hope for developing non-toxic and efficient anticancer therapies. Targeting of cancer cells relies on the development of molecular beacons, suited for in vivo applications, that are endowed with the required affinity, specificity, and favorable pharmacokinetic properties.

With the systematic evolution of ligands by exponential enrichment (SELEX) technology [1,2], specific macromolecular ligands—aptamers—can be generated by screening very large pools of oligonucleotides containing regions of random base composition with reiterated cycles of enrichment and amplification. At each cycle, the individual oligonucleotides with affinity for the desired target are kept, those with affinity for the sham target are rejected, and the population is enriched in oligonucleotides that distinguish between sham and real target. Aptamers that recognize a wide variety of targets, from small molecules to proteins and nucleic acids, and from cultured cells to whole organisms, have been described [3,4,5,6,7,8,9,10]. These oligonucleotides generally meet the requirements for in vivo diagnostic and/or therapeutic applications: Besides their good specificity and affinity, they are poorly immunogenic, and the SELEX technology can now accept chemically modified nucleotides for improved stability in biological fluids [11]. Conspicuously, less than fifteen years after the first applications of the technique, several lead compounds, including an anti-vascular endothelial growth factor aptamer [12], are currently under clinical trials [13].

Receptor tyrosine kinases (RTKs) are involved in a variety of signaling processes that regulate cell growth and proliferation and in several cancers [14]. RTKs are privileged targets for cancer therapy, which is underscored by the promising outcome of clinical trials with small molecules or antibody inhibitors [14]. In the present study, we validated a general strategy to target transmembrane receptors by SELEX. The RET (rearranged during transfection) RTK is physiologically

stimulated by any member of the glial cell line-derived neurotrophic factor (GDNF) family [15,16]. Germline mutations in the *RET* gene are responsible for constitutive activation of the receptor and for inheritance of multiple endocrine neoplasia (MEN) type 2A and 2B syndromes and of familial medullary thyroid carcinoma [17,18,19,20].

Mutations in the extracellular domain of RET, responsible for MEN2A syndrome, lead to constitutive dimerization of two mutated RET molecules. Conversely, a single point mutation, within the RET catalytic domain, that causes the MEN2B syndrome, involves an intramolecular mechanism to convert *RET* into a dominant transforming gene. Therefore, RET constitutes a model system of choice [20], in that the transforming mutations located in the extracellular domain simplify the issue of intracellular accessibility for a molecule targeting the receptor mutated in the extracellular domain (in its monomeric or dimeric form) and might provide alternative models (e.g., RET with mutations of the 2B kind) for controls or to elucidate the mode of target recognition.

Here we adopted a whole-cell SELEX strategy to target RET in a complex environment that is expected to expose a native

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Abbreviations: 2'-F-Py, 2'-fluoropyrimidine; ERK, extracellular signal-regulated protein kinase; GDNF, glial cell line-derived neurotrophic factor; GFR, GDNF family receptor $\alpha 1$; HER, heregulin; MEN, multiple endocrine neoplasia; NGF, nerve growth factor; RET, rearranged during transfection; RTK, receptor tyrosine kinase; SELEX, systematic evolution of ligands by exponential enrichment; VGF, nerve growth factor-inducible protein

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protein to the selection procedure, thus best mimicking in vivo conditions. We obtained aptamers that not only recognize the extracellular domain of RET, but also block RET downstream signaling and subsequent molecular and cellular events. The fact that aptamers with antioncogenic activity were isolated in the absence of a specific selective pressure suggests that our method could be used to identify active macromolecules with potential therapeutic interest against other transmembrane receptors.

Results

A library of 2'-fluoropyrimidine (2'-F-Py), nuclease-resistant RNAs was subjected to a differential SELEX protocol against intact cells expressing different forms of the human *RET* oncogene (Figure 1). For the selection step, PC12 cells were used that express the human RET^{C634Y} mutant receptor (PC12/MEN2A). RET^{C634Y} is mutated in the extracellular domain and forms spontaneously active homodimers on the cell surface, which induces biochemical and morphological changes that mirror the RET-dependent human pheochromocytoma phenotype of MEN2 syndromes [21]. The counter-selection necessary to avoid selecting for aptamers that nonspecifically recognized the cell surface included a first step against parental PC12 cells in order to eliminate nonspecific binders of the PC12 cell surface, followed by a second counterselection step against PC12/MEN2B cells that expressed an allele of RET (RET^{M918T}) mutated in the

intracellular tyrosine kinase domain. PC12/MEN2B and PC12/MEN2A cells have a similar morphology, but the extracellular domain of the RET^{M918T} receptor is identical to the wild type and, in the absence of the ligand and co-receptor, remains monomeric. This step was originally aimed at selecting aptamers that recognize specifically the dimeric form of the extracellular domain.

After 15 rounds of selection, the pool of remaining sequences bound PC12/MEN2A cells in a saturable manner with an apparent K_d approximating 100 nM. From this pool, 67 sequences were cloned and analyzed. Two individual sequences (D14 and D12) dominated the selection and constituted together more than 50% of the clones, four other sequences represented together 25% of the clones, and eight sequences were present only once. As is often the case for a selection against a complex target [7,22] (and in contrast to in vitro SELEX on purified proteins) we found almost no similarity among sequences, except for clones D24 and D4, which shared common sequence motifs and structure prediction (Figure 2A).

We assessed binding to PC12/MEN2A cells of all individual aptamers that were found more than once and also of some unique sequences (including D4 and D24). Several sequences bound PC12/MEN2A cells with apparent K_d values ranging from 30 to 70 nM (Figure 2B and unpublished data), but not parental PC12, rat-derived bladder carcinoma (NBTII), or human cervical carcinoma (HeLa) cells (Figure 2C and unpublished data). As a first attempt to deconvolute the complex pool of winning aptamers, we first produced a recombinant fragment of RET, EC- RET^{C634Y} [23], but all attempts to identify in the winning pool aptamers binding to EC- RET^{C634Y} were fruitless. Likewise, SELEX against this purified EC- RET^{C634Y} protein gave rise to aptamers unable to recognize the PC12/MEN2A cells, suggesting that they did not bind to the RET protein present in its native conformation on the cell surface. Consequently, we screened the winning pool of aptamers for the ability to interfere with the biological activity of RET. To this end, we used an in vitro cell system in which we assessed the capability of each aptamer to inhibit RET^{C634Y} autophosphorylation and receptor-dependent downstream signaling. Mutant RET^{C634Y} , expressed in PC12/MEN2A cells, forms homodimers on the cell surface that cause constitutive activation of its tyrosine kinase activity [24] and induce several downstream signaling cascades, including the activation of extracellular signal-regulated protein kinase (ERK) [25]. As previously reported [25], levels of phosphorylated RET and ERK were constitutively high in untreated PC12/MEN2A cells due to the presence of the active RET^{C634Y} allele. Surprisingly, some of the tested aptamers inhibited RET^{C634Y} and ERK phosphorylation, compared to the control starting pool and to the other aptamers (Figure 3A and unpublished data). In all experiments, inhibition of phosphorylation was more rapid and quantitative for ERK than for RET^{C634Y} . We believe that this is due to a different sensitivity to changes in RET tyrosine kinase activity of the two processes and/or to differences in the half-lives of the phosphorylated forms of the two proteins [26]. In a dose-response experiment (Figure 3B, left panel), the best inhibitor, D4, was effective at a concentration of 200 nM to inhibit RET^{C634Y} autophosphorylation up to 70% and to drastically reduce ERK phosphorylation. Time-activity studies showed that the treatment of PC12/MEN2A cells at

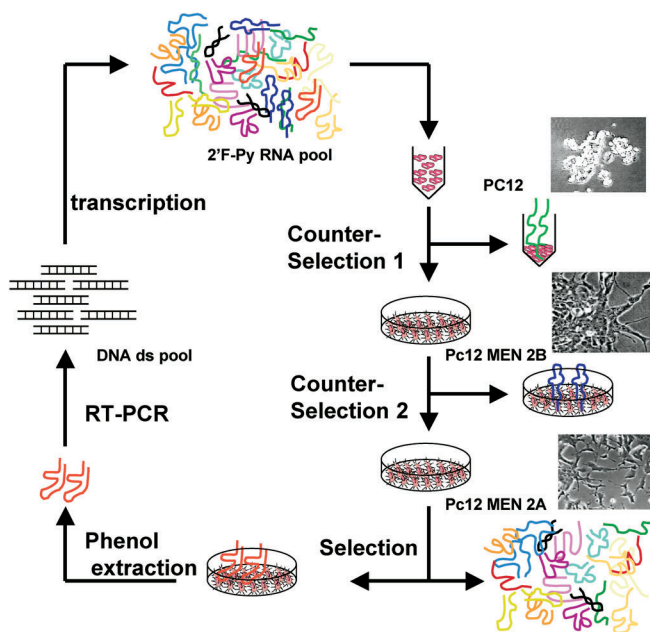


Figure 1. Schematic Protocol for the Selection of PC12/MEN2A Cell-Specific Aptamers

A pool of 2'-F-Py RNAs was incubated with suspended parental PC12 cells (Counterselection 1). Unbound sequences were recovered by centrifugation and incubated with adherent PC12/MEN2B cells (Counterselection 2). Unbound sequences in the supernatant were recovered and incubated with adherent PC12/MEN2A cells for the selection step (Selection). Unbound sequences were discarded by several washings, and bound sequences were recovered by phenol extraction. Sequences enriched by the selection step were amplified by RT-PCR and in vitro transcription before a new cycle of selection. DOI: 10.1371/journal.pbio.0030123.g001

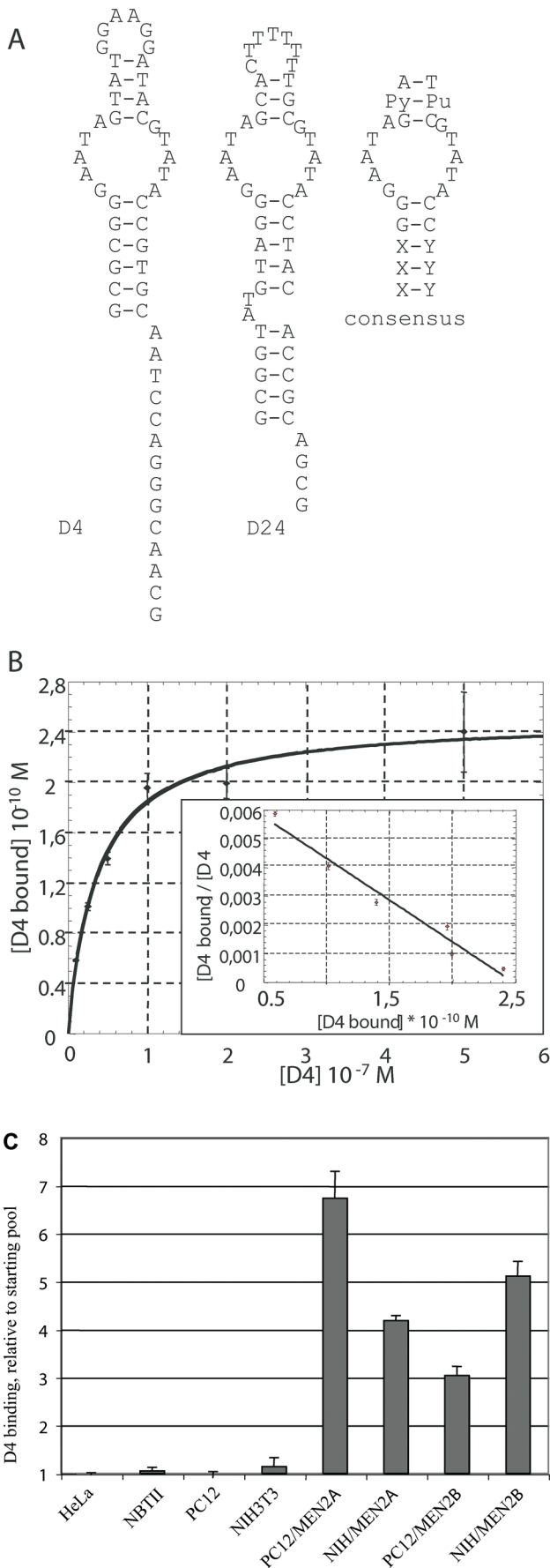


Figure 2. Predicted Structure and Association Constants of D4 and D24 (A) Comparison of a secondary structure prediction for the D4 and D24 aptamers. Structures were predicted using MFOLD software version 3.1 (available at <http://www.bioinfo.rpi.edu/applications/mfold/>). (B) Binding curve of the D4 aptamer on PC12/MEN2A. D4 was ³²P-radiolabeled and incubated at different concentrations on cell monolayers. The background binding value for a D4 scrambled sequence is subtracted from every data point. Scatchard analysis (inset) was used for the evaluation of the binding constant. (C) Binding of the ³²P-labeled D4 aptamer to several cell lines expressing (or not) human RET. Binding was performed on the cell lines indicated in the same condition at 50 nM, and the results are expressed relative to the background binding detected with the starting pool of sequences used for selection. Expression of RET could not be detected by Western blot in HeLa, NBTII, PC12wt and NIH3T3 cells, whereas PC12/MEN2A and NIH3T3/MEN2A express RET^{C634Y} and PC12/MEN2B and NIH3T3/MEN2B express RET^{M918T}. DOI: 10.1371/journal.pbio.0030123.g002

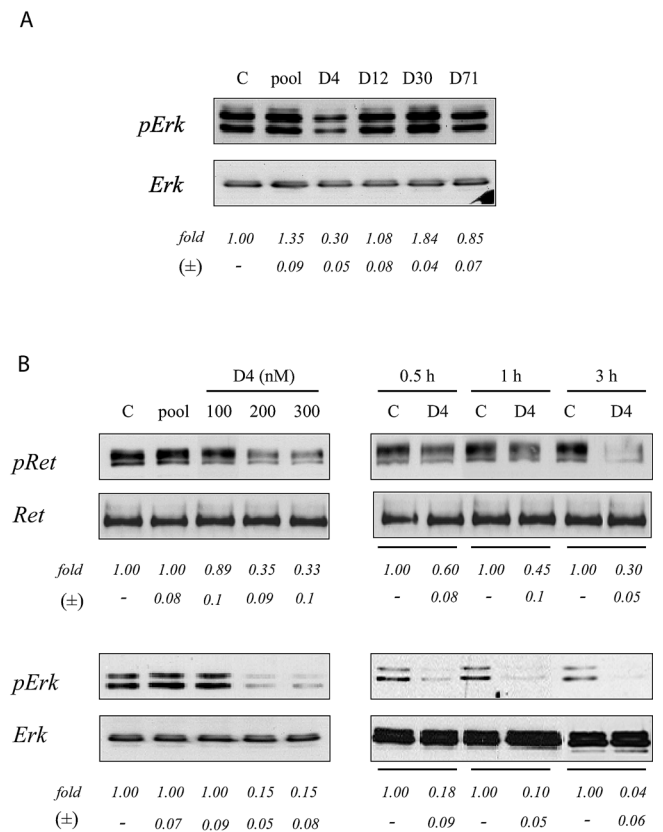


Figure 3. Effect of Selected Aptamers on RET^{C634Y} Activity (A) PC12/MEN2A cells were either left untreated or treated for 16 h with 150 nM of the indicated RNA aptamer, or the starting RNA pool (pool). Cell lysates were immunoblotted with anti-(phospho)-ERK (pErk), then stripped and reprobbed with anti-ERK (Erk) to confirm equal loading. Values below the blots indicate signal levels relative to untreated controls. (B) PC12/MEN2A cells were treated for 1 h with increasing amounts of D4 (left blots) or with 200 nM D4 for the indicated incubation times (right blots). Cell lysates were immunoblotted with anti-(Tyrosylphosphorylated)-RET (pRet) or anti-(phospho)-ERK (pErk) antibodies, as indicated. To confirm equal loading the filters were stripped and reprobbed with anti-RET (Ret) or anti-ERK (Erk) antibodies, respectively. In (A) and (B), “C” indicates mock-treated cells. Quantitations were done on the sum of the two RET- or ERK- specific bands, and values are expressed relative to the control, arbitrarily set to 1. Standard deviations are indicated (*n* = 4). DOI: 10.1371/journal.pbio.0030123.g003

200 nM for 1 h was sufficient to significantly inhibit RET^{C634Y} autophosphorylation and to drastically reduce ERK phosphorylation (Figure 3B, right panel).

Comparison of the predicted structures of D4 and of the related clone D24 (Figure 2A) suggests that a conserved stem-internal loop-stem is crucial for binding. Consistently, we found that replacing the apical loop with a stable tetraloop (UUGC) or deleting nucleotides not included in the conserved structure did not significantly affect binding of D4 to PC12/MEN2A cells (unpublished data). However, only the full-length D4 inhibits RET^{C634Y} signaling, demonstrating that binding is necessary but not sufficient for inhibition. A 2′F-Py RNA oligonucleotide of identical composition but with a scrambled sequence (D4Sc) was ineffective for both binding and inhibition.

The D4 aptamer bound to PC12/MEN2A with an estimated apparent K_d of 35 ± 3 nM (Figure 2B), but also to PC12/MEN2B cells (Figure 2C and unpublished data), suggesting that one of the counterselection steps employed in the SELEX procedure was ineffective in this case. The D4 aptamer bound to transfected NIH3T3 cells expressing at similar levels the two mutant forms (RET^{C634Y} and RET^{M918T}) of the RET receptor (NIH/MEN2A and NIH/MEN2B, respectively [Figure 2C; see also below]). Binding was dependent on expression of human RET, as D4 did not recognize parental untransfected PC12, NIH3T3 cells, or other cell lines, including rat NBTII, human HeLa cells, and mouse MN1 (Figure 2C and unpublished data). Interestingly, the latter, a mouse motor

neuron-neuroblastoma fusion cell line, expresses the mouse RET^{wt}, suggesting some species-specificity in RET recognition by D4. Finally, D4 bound a human neuroblastoma cell line (SK-N-BE) that naturally expresses endogenous RET (L. Cerchia et al., personal communication). Consistently with what was observed for the pool of winning aptamers, D4 was unable to bind the purified EC-RET^{C634Y} protein (unpublished data), thus supporting the specificity for the membrane-bound RET.

We next determined whether D4 could inhibit wild-type RET. Cells from a PC12-derived cell line expressing the human wild-type RET (PC12/wt) were stimulated with a mixture containing GDNF and soluble GDNF family receptor α1 (GFRα1), and either treated with the D4 aptamer or with the starting pool of 2′F-Py RNA as a negative control. As shown in Figure 4A, the D4 aptamer, but not the control RNA pool, strongly inhibited GDNF-induced phosphorylation of RET (left panel) and of the downstream effector ERK (middle panel). A similar inhibitory effect was observed in PC12-α1/wt cells, a PC12-derived cell line that stably expresses both human RET and GFRα1 (unpublished data). In contrast, D4 was inactive in inhibiting the signaling triggered by the unrelated nerve growth factor (NGF) receptor tyrosine kinase TrkA, thus indicating that D4-induced inhibition of ERK phosphorylation was specific for RET intracellular signaling (Figure 4A, right pane)

Although the D4 aptamer binds PC12/MEN2B cells, treating these cells with 200 nM D4 for 1 h (Figure 4B) or longer,

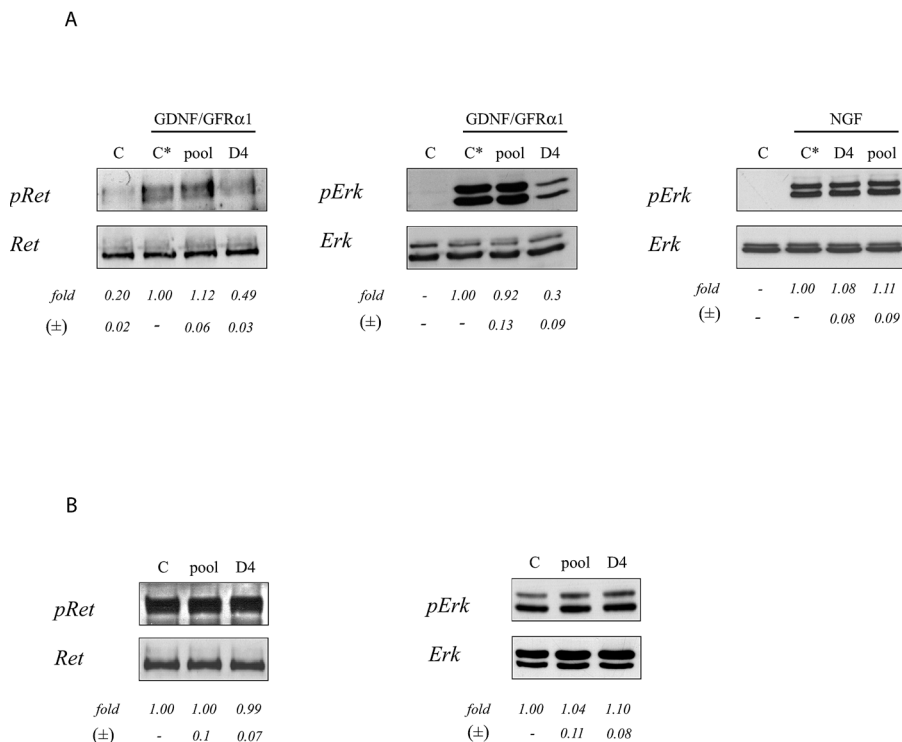


Figure 4. D4 Aptamer Inhibits RET^{wt} but Not RET^{M918T} Activity

(A) PC12/wt cells were treated for 10 min with GDNF (50 ng/ml) and soluble GFRα1 (1.6 nM), or 5 min with NGF (100 ng/ml), together with 200 nM of either the D4 aptamer or the starting RNA pool. “C*” indicates cells treated with GDNF and GFRα1 in the absence of aptamer.

(B) PC12/MEN2B cells were starved for 6 h and then treated for 1 h with 200 nM D4 or the starting RNA pool. Cell lysates were immunoblotted with anti-(Tyr-phosphorylated)-RET or anti-(phospho)-ERK antibodies, as indicated (see Figure 3 legend).

In (A) and (B), “C” indicates mock-treated cells. Quantitations were done as in Figure 3, and relative abundances are expressed relative to controls, arbitrarily set to 1. Standard deviations are indicated ($n = 4$).

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or at higher D4 concentrations (unpublished data), did not interfere with signaling due to the monomeric RET^{M918T}. This further confirms that inhibition of ERK phosphorylation is not a nonspecific effect of exposing the cells to the D4 aptamer. The kinase and the biological activities of RET^{M918T}, although constitutive, are responsive to GDNF stimulation in the presence of GFR α 1 [27,28]. Similarly to the inhibition of RET^{wt} activity, the treatment of PC12/MEN2B cells by D4 abolished the GDNF-dependent overstimulation of RET and ERK phosphorylation (unpublished data). These data strongly suggest that D4 inhibits exclusively the dimerization-dependent RET activation.

We then searched for phenotypic effects of D4 on RET-dependent cell differentiation and transformation. First we measured neurite outgrowth in PC12- α 1/wt cells following GDNF stimulation. As shown in Figure 5, cells extended long neurite-like processes in response to a 48-h exposure to

GDNF (Figure 5B) with respect to the nonstimulated control cells (Figure 5A). Treatment of the cells with the D4 aptamer (Figure 5C), but not with the D4Sc scrambled control (Figure 5D), significantly decreased the proportion of neurite outgrowth (Figure 5E). To biochemically monitor differentiation, we determined the levels of the nerve growth factor-inducible protein (VGF) in cell extracts following 48 h of treatment. VGF is an early gene that is rapidly induced by both NGF and GDNF in PC12 cells [29]. As expected, in GDNF-treated cells, VGF expression was stimulated and, consistent with the phenotypic effects reported above, treatment with D4, but not with D4Sc, kept the VGF levels close to basal (Figure 5F).

Upon expression of either RET^{C634Y} or RET^{M918T}, NIH3T3 cells show drastic changes in their morphology [24]. We treated NIH/MEN2A and NIH/MEN2B cells stably expressing the RET mutants with D4 for 72 h, and analyzed the

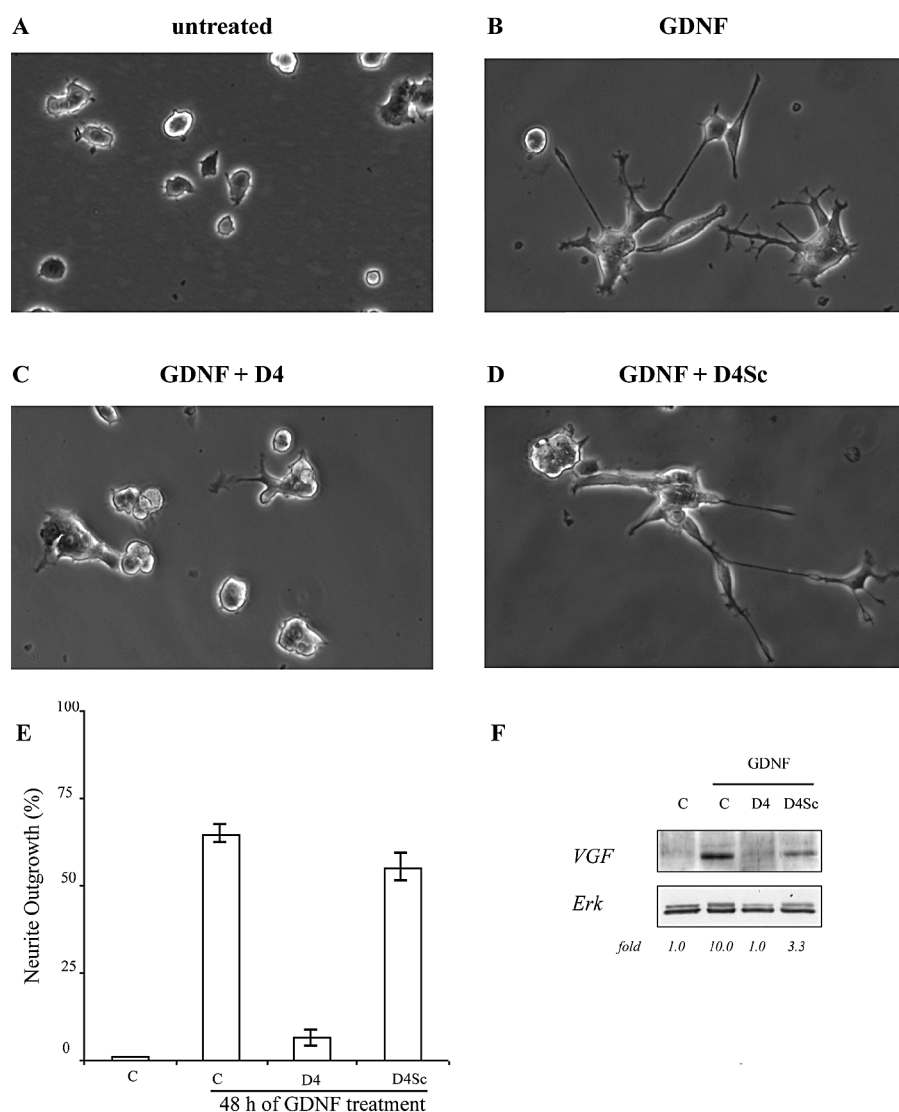


Figure 5. D4 Aptamer Inhibits the GDNF-Induced Differentiation of PC12- α 1/wt Cells

Cells were either left unstimulated (A), stimulated with GDNF (B), or with GDNF together with D4 or D4Sc (C and D, respectively). Following 48 h of GDNF treatment, the percentage of neurite outgrowth was calculated. The data represent the average of three independent experiments and are expressed as percentage of neurite-bearing cells/total cells analyzed (E). Following 48 h of treatment, cells were lysed and proteins immunoblotted with anti-VGF antibodies. Equal loading was confirmed by immunoblotting with anti-ERK antibodies as indicated (F).

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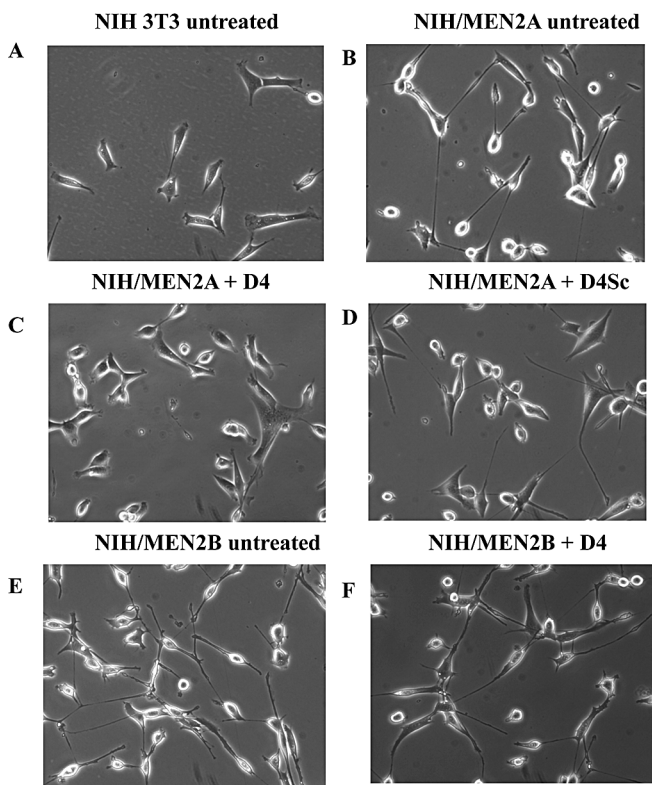


Figure 6. D4 Aptamer Reverts the Transformed Morphology of NIH/MEN2A Cells

NIH3T3-derived cell lines were either left untreated (A, B, and E) or treated with D4 (C and F) or D4Sc (D), and the cells were maintained in culture for 72 h. Each experiment was repeated a minimum of three times.

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morphological changes induced by the aptamer. As shown in Figure 6, NIH/MEN2A and NIH/MEN2B cells have a spindle shape, long protrusions, and a highly refractive appearance (Figure 6B and 6E, respectively). As expected, D4-treated NIH/MEN2A cells (Figure 6C) reverted to a flat and polygonal morphology similar to the parental NIH3T3, whereas no morphological changes were observed in NIH/MEN2B (Figure 6F), which is consistent with the notion that constitutive signaling from RET^{C634Y}, but not from RET^{M918T}, is inhibited by D4. On the other hand, treatment with D4Sc had no effects on any cell line (Figure 6D and unpublished data).

Discussion

RTKs are involved in a variety of signaling pathways that affect cell growth and differentiation. Targeting specifically RTKs holds potential for dissecting the molecular mechanisms of receptor function, but also for diagnosis and therapeutics of cancer [14].

Here we employed a modified SELEX procedure to target the RET RTK, and we obtained nuclease-resistant RNA ligands capable of binding and inhibiting the protein on the cell surface. Aptamers against recombinant heregulin 3 (HER3) RTK have been recently isolated and shown to inhibit the heregulin-induced activation of the HER3/HER2 dimer [30]. However, finding the most efficient binders and

inhibitors is likely to generally rely on the recognition of the target protein in its native state.

In the case of transmembrane receptors, whole-cell SELEX offers the advantage of selecting molecules capable of recognizing the target protein in its natural glycosylation state and presented in its physiological environment. An important drawback of this strategy is the lack of knowledge of the identity and abundance of the effective targets and the possibility that unwanted aptamers may dominate the selection, preventing the emergence of the molecules of interest. However, the abundance of the target protein and an appropriate selection scheme might provide sufficient selective pressure to favor the wanted aptamers [10].

The D4 aptamer binds to different cell types, provided that human RET is expressed on the cell surface, and specifically inhibits both RET and ERK phosphorylation, strongly suggesting that RET is the bona fide target of D4. Interestingly, aptamers isolated by whole-cell SELEX were unable to bind purified EC-RET^{C634Y} and, conversely, aptamers coming from the selection with purified EC-RET^{C634Y} were unable to bind the membrane-bound RET. Thus, it is likely that D4 binding is dependent on the association of RET with the cellular membrane, which might reflect changes in the receptor's conformation/modification state or, alternatively, might imply unidentified molecular components interacting with RET at the cell surface. This latter possibility is supported by a recent report demonstrating that the presence of heparan sulfate glycosaminoglycan on the cell surface is required for RET-dependent GDNF intracellular signaling [31].

Our interpretation of the D4 aptamer's mode of action relies upon three observations: (1) D4 binds with similar affinities to cells expressing RET in a monomeric or dimeric form; (2) D4 inhibits dimerization-dependent RET activation, as a consequence either of GDNF stimulation of RET^{wt} or RET^{M918T} or of constitutive dimerization of the RET^{C634Y} mutant; and (3) D4 does not inhibit a monomeric form of RET that is constitutively activated by a mutation in the intracellular kinase domain (RET^{M918T}). These results taken together are compatible with the notion that D4 acts by interfering with the formation of a stable, active RET dimer, regardless of whether dimerization is caused by the formation of the RET/GDNF/GFR α 1 complex or by the direct interaction of two mutated RET^{C634Y} proteins. This might occur either by D4 binding to monomeric RET, which would impede subsequent formation of the dimer, or by binding directly to the dimer.

Differential whole-cell SELEX strategies (this work; see also [5,7,8,10]) can be employed to identify new markers on the surface of a given cell type, define the specificity of a cellular state, and/or allow in vivo targeting for diagnostic and therapeutic applications. The identification of lead compounds by reiterated affinity selection on living cells appears crucial when the molecular target is a membrane-bound or large transmembrane protein for which the conformation is frequently dictated by the interaction with other molecules, including membrane constituents [31]. Given that several of these proteins, as transmembrane receptors, integrins, and adhesion molecules, are involved in cell proliferation, apoptosis, and differentiation, aptamers for these targets could be promising prognostic tools in human therapy for

widespread, devastating diseases such as cancer and neurodegeneration.

Materials and Methods

Cell culture and immunoblot analysis. Growth conditions for PC12 cells and derived cell lines were previously described [32]. NIH/MEN2A and NIH/MEN2B cells were obtained from NIH3T3 cells stably transfected with vectors expressing human RET^{C634Y} and RET^{M918T}. To assess the effects of aptamers on RET activity, cells (160,000 cells per 3.5-cm plate) were serum-starved for 2 h and then treated with the indicated amount of RNA aptamers or the starting RNA pool after a short denaturation-renaturation step. When indicated, 2.5S NGF (Upstate Biotechnology, Lake Placid), GDNF (Promega), or recombinant rat GFR α 1-Fc chimera (R&D Systems, Minneapolis, Minnesota, United States) were added to the culture medium. Cell extracts and immunoblotting analysis were performed as described [23]. The primary antibodies used were anti-RET (C-19), anti-VGF (R-15), and anti-ERK1 (C-16) (all three, Santa Cruz Biotechnology, Santa Cruz, California, United States); and anti-(Tyr-phosphorylated) RET and anti-phospho-44/42 MAP kinase (also indicated as anti-[phospho]-ERK) monoclonal antibodies (E10) (both from Cell Signaling, Beverly, Massachusetts, United States). Four independent experiments were performed.

Cell transformation and neurite outgrowth bioassay. PC12- α 1/wt or NIH3T3 cells were plated at equal density on 12-well culture plates. Aptamers were added at 3 μ M final concentration to the growth medium. To ensure the continuous presence of a concentration of at least 200 nM, this treatment was renewed every 24 h, which takes into account the half-life of the D4 aptamer in 10% serum (approximately 6 h, unpublished data). At least 15 random fields were photographed every 24 h with a phase-contrast light microscope. To evaluate the effects of D4 on cell differentiation, cells were pretreated for 6 h with 400 nM D4 or D4Sc and then incubated with 50 ng/ml GDNF together with 3 μ M of the appropriate aptamer (see above). At 24 and 48 h of GDNF stimulation, 50 cells per frame were counted and scored as having neurites or not. A neurite was operationally defined as a process outgrowth with a length more than twice the diameter of cell body.

Ex vivo SELEX. The SELEX cycle was performed essentially as described [33]. Transcription was performed in the presence of 1 mM 2'F-Py and a mutant form of T7 RNA polymerase (T7^{Y639F}, kind gift of R. Souza) [11] was used to improve yields. 2'F-Py RNAs were used because of their increased resistance to degradation by seric nucleases. The complexity of the starting pool was roughly 10¹⁴. 2'F-Py RNAs (1–5 nmol) were heated at 85 °C for 5 min in 3 ml of RPMI 1640, snap-cooled on ice for 2 min, and allowed to warm up to 37 °C before incubation with the cells. Two counterselection steps were performed per cycle. To avoid selecting for aptamers nonspecifically recognizing the cell surface, the pool was first incubated for 30 min at 37 °C with 10⁷ PC12 cells, and unbound sequences were recovered by centrifugation. These were subsequently incubated with 10⁷ adherent PC12/MEN2B cells, expressing a human RET receptor mutated in the intracellular domain (RET^{M918T}), and unbound sequences were recovered for the selection phase. This step was meant to select sequences recognizing specifically the human RET receptor mutated in the extracellular domain (RET^{C634Y}) expressed on PC12/MEN2A cells. The recovered sequences were incubated with 10⁷ adherent PC12/MEN2A cells for 30 min at 37 °C in the presence of nonspecific competitor RNA (total yeast RNA) and recovered after several washings with 5 ml of RPMI by total RNA extraction (Extract-All, Eurobio, Les Ulis, France).

During the selection process, we progressively increased the selective pressure by increasing the number of washings (from one

for the first cycle up to five for the last three cycles) and the amount of nonspecific RNA competitor (100 μ g/ml in the last three cycles), and by decreasing the incubation time (from 30 to 15 min from round 5) and the number of cells exposed to the aptamers (5 \times 10⁶ in the last three cycles). To follow the evolution of the pool we monitored the appearance of four-base restriction sites in the population, which reveals the emergence of distinct families in the population [34]. After 15 rounds of selection, sequences were cloned with TOPO-TA cloning kit (Invitrogen, Carlsbad, California, United States) and analyzed.

Binding experiments. Binding of individual aptamers (or the starting pool as a control) to PC12 cells and derivatives was performed in 24-well plates in triplicate with 5'-³²P-labeled RNA. 10⁵ cells per well were incubated with various concentrations of individual aptamers in 200 μ l of RPMI for 10 min at 37 °C in the presence of 100 μ g/ml polyinosine as a nonspecific competitor. After extensive washings (5 \times 500 μ l of RPMI), bound sequences were recovered in 350 μ l of SDS 0.6%, and the amount of radioactivity recovered was normalized to the number of cells by measuring the protein content of each well. Binding of individual sequences to different cell lines was performed in the same condition at 50 nM only.

For the binding curve of D4 to PC12/MEN2A cells (see Figure 2B), nonspecific binding was assessed using a 5'-³²P-labeled naive pool of 2'F-RNAs (i.e., the starting pool of the selection), and the background values obtained were subtracted from the values obtained with the D4 aptamer. Apparent K_d values for each aptamer were determined by Scatchard analysis according to the equation

$$\frac{[\text{bound aptamer}]/[\text{aptamer}]}{+ ([T]_{\text{tot}}/K_d)} = \frac{-(1/K_d) \times [\text{bound aptamer}]}{+ ([T]_{\text{tot}}/K_d)} \quad (1)$$

where [T]_{tot} represents the total target concentration.

Supporting Information

Accession Numbers

The Swiss-Prot (<http://www.ebi.ac.uk/swissprot/>) accession numbers for the proteins discussed in this paper are ERK (P27361), GDNF (P39905), GFR α 1 (P56159), NGF (P01138), RET RTK (P07949), TrkA (P04629), and VGF (P20156).

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Competing interests. A patent application was filed covering the D4 aptamer and its use in diagnostic and therapeutics of cancer.

Author contributions. LC, FD, BT, VdF, and DL conceived and designed the experiments. LC, FD, CP, JB, YA, and KG performed the experiments. LC, FD, BT, VdF, and DL analyzed the data. VdF and DL contributed reagents/materials/analysis tools. BT, VdF, and DL wrote the paper. ■

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