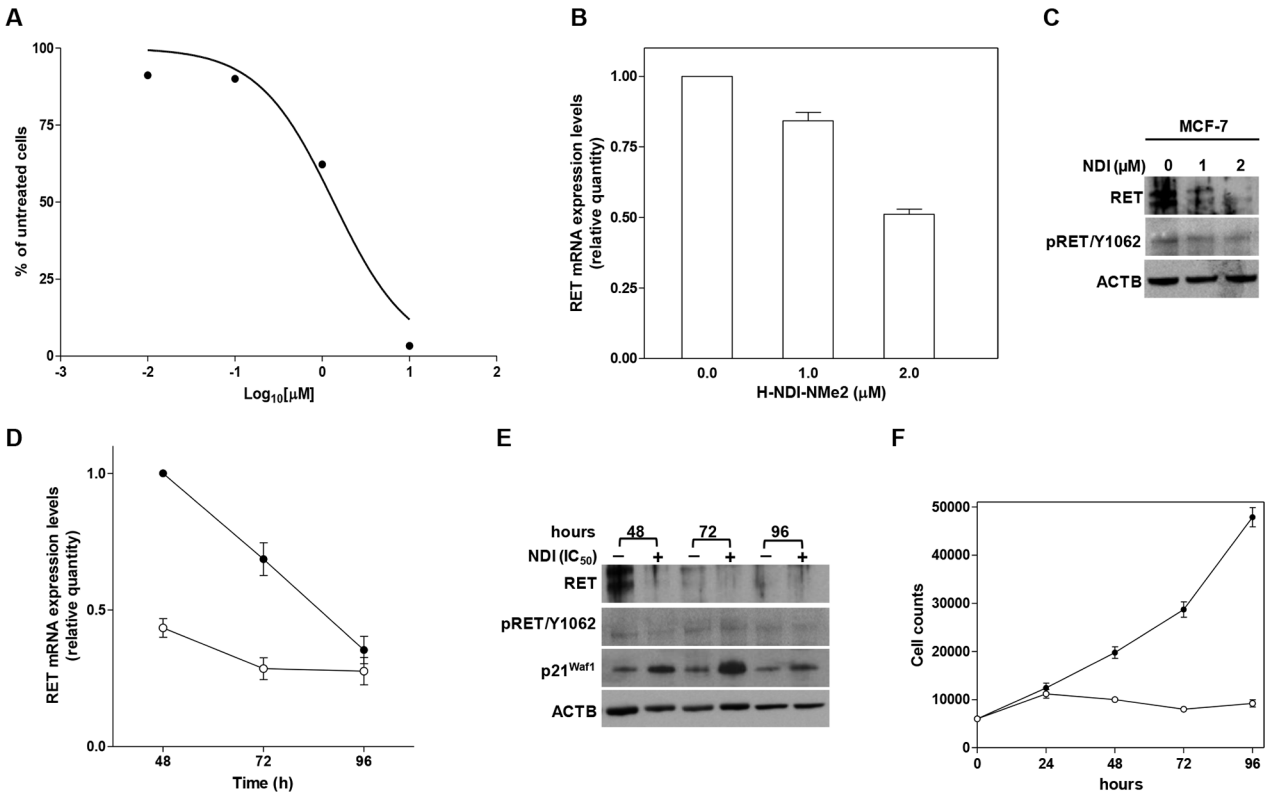
Targeting of *RET* oncogene by naphthalene diimide-mediated gene promoter G-quadruplex stabilization exerts anti-tumor activity in oncogene-addicted human medullary thyroid cancer

SUPPLEMENTARY MATERIALS AND METHODS

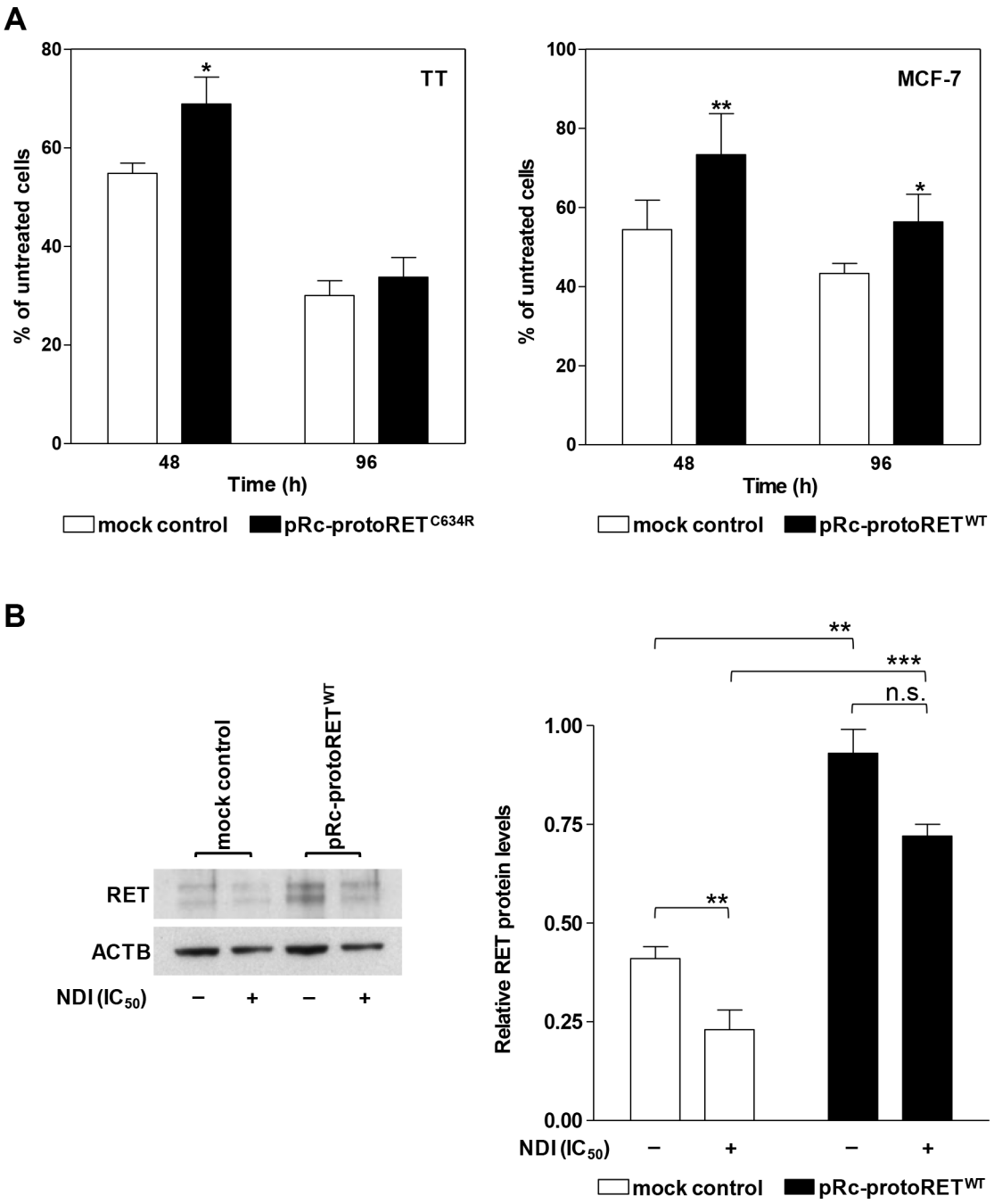
Transfection experiments

Transient transfection of RET encoding vectors was carried out according to standard procedures. Briefly, wild-type and MEN2A (C634R)-associated RET pRc/CMVconstructs were obtained as previously described [38]. Twenty-four (MCF-7) or 72 (TT) h after seeding in a 6-well plate, cells ($0.3x10^6$ cells/well) were transiently transfected with 2.5 μg/well of pRC/CMV plasmid (Thermo Fisher Scientific, Monza, Italy) encoding wild-type or mutant (C634R) protoRET using Lipofectamine® 3000 (Thermo Fischer Scientific) in standard growth medium containing 10% FBS, according to the manufacturer's protocol. Forty-eight hours after transfection, cells were treated with the NDI derivative for 48 and 96 h at a concentration corresponding to the IC_{50}. Cells were then collected, counted in a particle counter and subsequently analysed.

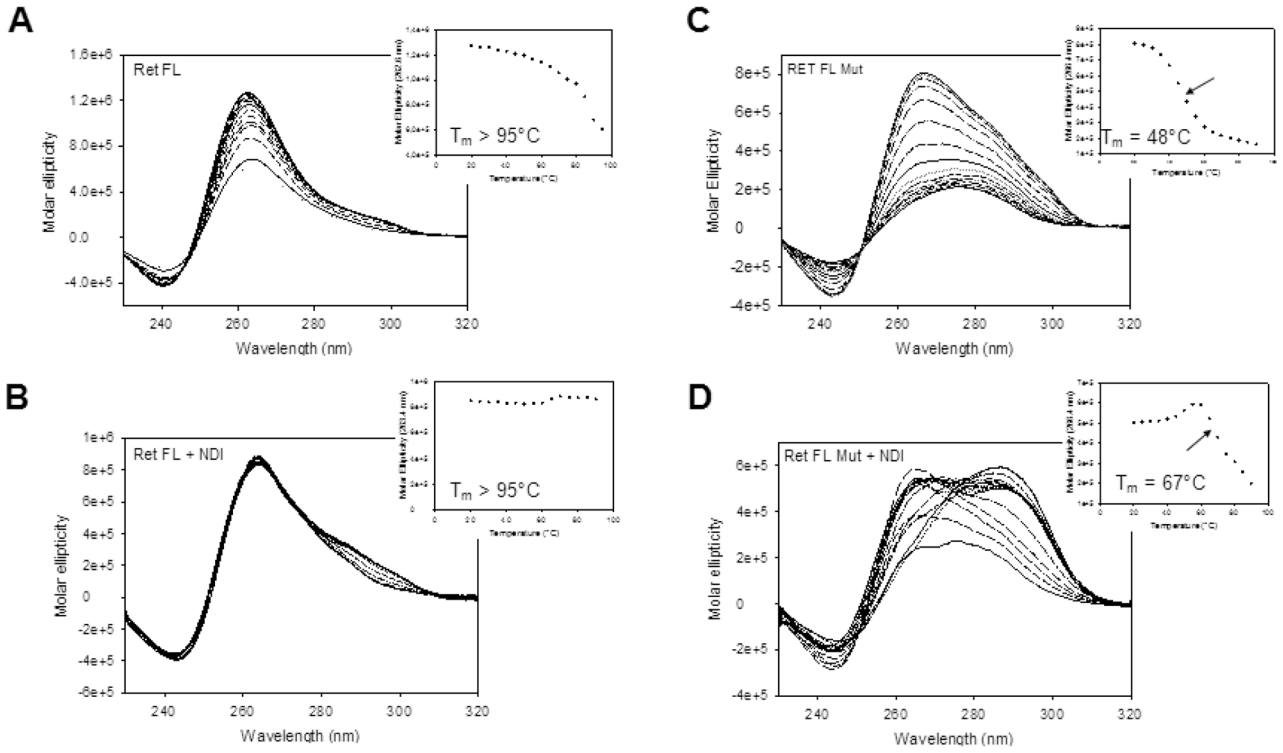
SUPPLEMENTARY FIGURES AND TABLES



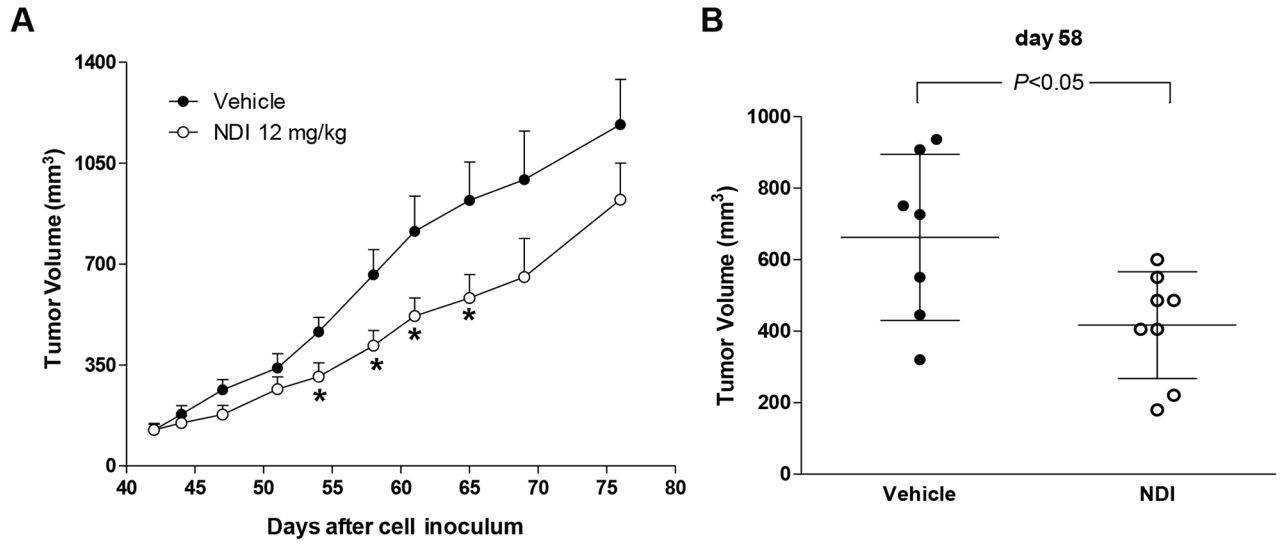
Supplementary Figure S1: Exposure of MCF-7 breast cancer cells to NDI resulted in the impairment of cell growth and in the down-regulation of RET expression. A. Cell growth inhibition curve obtained upon a 48-h exposure to increasing concentrations (Log_{10} [μM]) of NDI. Data have been reported as percentage of growing cells in treated *vs.* untreated cells and represent mean values; **B.** Real-time RT-PCR assessment of RET mRNA expression in MCF-7 cells exposed for 24 h to increasing concentrations of NDI. Quantification of RET mRNA levels was carried out according to the $2^{-\Delta\Delta Ct}$ method [39]. Data have been reported as RET mRNA relative quantity in treated *vs.* untreated cells and represent mean values ± s.d.; **C.** Representative western immunoblotting showing basal and phosphorylated (pRET/Y1062) RET protein amounts in cells exposed for 24 h to the indicated concentrations of NDI. β-actin (ACTB) was used to ensure equal protein loading; **D.** Time-course assessment of RET mRNA expression levels in untreated (●) and NDI-treated (○) cells. Data have been reported as RET mRNA relative amounts in untreated and NDI-treated cells, according to $2^{-\Delta C T}$ method [39], and represent mean values ± s.d.; **E.** Representative western immunoblotting showing the time-course assessment of basal and phosphorylated (pRET/Y1062) RET as well as of $p21^{waf1}$ protein amounts in untreated and NDI-treated MCF-7 cells; **F.** Time-course evaluation of cell growth in untreated (●) and NDI-treated (○) breast cancer cells. Data have been reported as number (cell counts) of growing cells and represent mean values ± s.d..



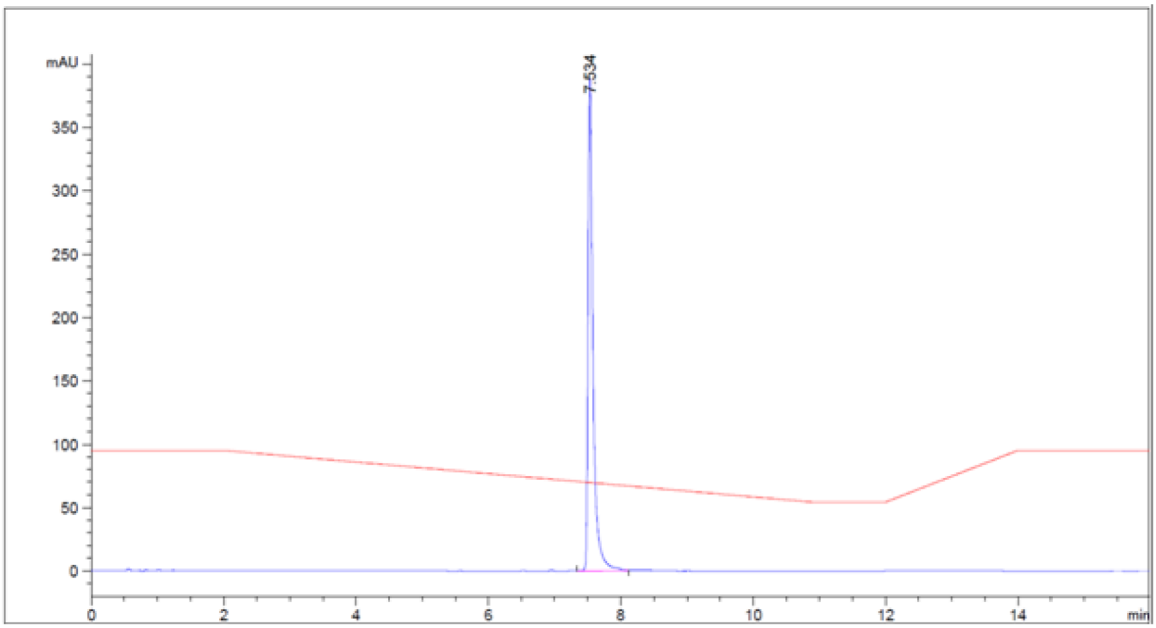
Supplementary Figure S2: The ectopic expression of RET partially counteracts the growth inhibitory effects of NDI in MTC and BCa cells. A. TT (left panel) and MCF-7 (right panel) cells transfected with empty pRc/CMV vector (mock control) and MEN2A-associated (pRc-protoRET^{C634R}) or wild-type (pRc-protoRET^{WT}) RET-encoding vectors were exposed for 48 and 96 h to NDI (IC_{50}). Data have been reported as percentage of growing cells in NDI-treated *vs.* untreated cells and represent mean values ± s.d. **P*<0.05; ***P*<0.01; **B.** Representative western immunoblotting showing the expression levels of RET in mock control and pRc-protoRET^{WT}–transfected MCF-7 cells in the absence or presence of NDI (IC_{50}). β-actin (ACTB) was used to ensure equal protein loading. The quantification of relative RET protein levels in control and pRc-protoRET^{WT}–transfected MCF-7 cells has been reported in the graph on the right. Data represent mean values ± s.d. ***P*<0.01; ***P<0.001.



Supplementary Figure S3: CD thermal unfolding analysis of *RET* promoter G4 sequences. A. and **B.** CD spectra of the RET FL oligonucleotide at increasing temperature (25-95 °C) in the absence A) or presence B) of NDI. The insets show molar ellipticity at the indicated wavelength as a function of temperature. **C.** and **D.** CD spectra of the RET FL Mut oligonucleotide at increasing temperature (25-95 °C) in the absence C) or presence D) of the NDI. The insets show molar ellipticity at the indicated wavelength as a function of temperature. T_m values are indicated in the insets.



Supplementary Figure S4: The NDI derivative inhibits MTC tumor growth *in vivo*. A. Growth curves of TT tumors in vehicle-treated mice (●) and upon i.p. administration of 12 mg/kg NDI derivative (○). Data have been reported as average tumor volume (mm^3) ± S.E.M. *P<0.05. **B.** Tumor volume distribution in vehicle- and NDI-treated animals at day 58, at which the maximum TVI% was observed.

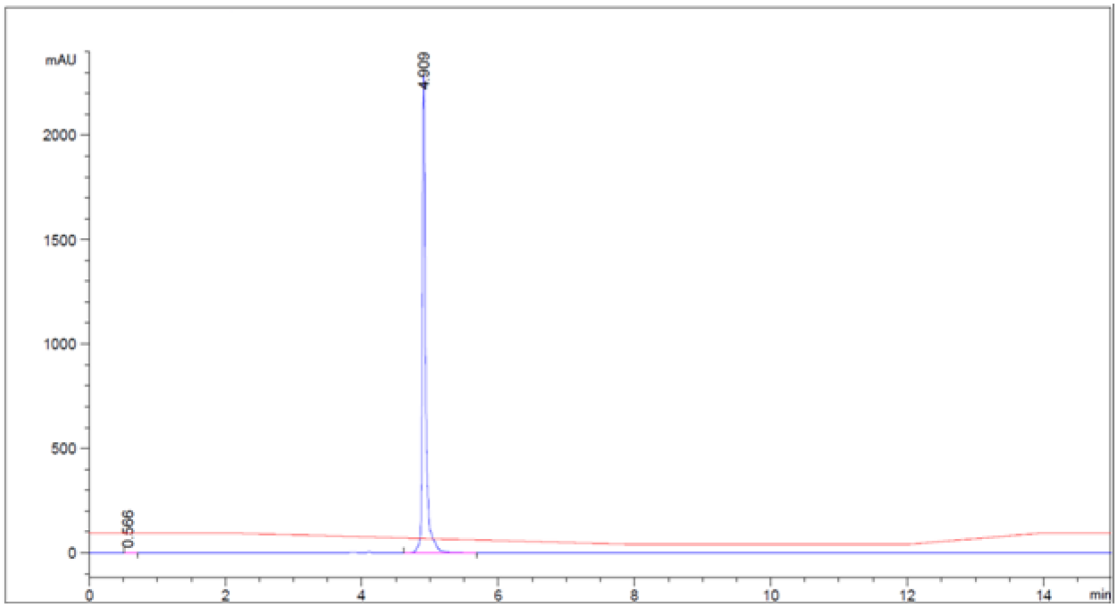


Signal 1: VWD1 A, Wavelength= 256 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	7.534	VV	0.0755	2013.92944	388.64218	100.0000
Totals	:			2013.92944	388.64218	

Time	% H_2O TFA 0.1%	% CH_3CN
	95	5
2	95	5
11	54.5	45.5
12	54.5	54.5
14	95	5
16	95	5

Supplementary Figure S5: HPLC purity data of the NDI derivative. The analytical chromatogram of a NDI sample assessed by Method-1 has been reported. HPLC flux 1.0 ml/min.



Signal 1: VWD1 A, Wavelength= 256 nm

Peak #	RetTime [min]	Type	Width [min]	Area [mAU*s]	Height [mAU]	Area %
1	0.566	BB	0.0387	5.39401	2.01595	0.0677
2	4.909	VV	0.0521	7959.05518	2287.87793	99.9323
Totals	:			7959.05518	2287.87793	

Time	% H_2O TFA 0.1%	% CH_3CN
	95	5
2	95	5
8	40	60
12	40	60
14	95	5
16	95	5

Supplementary Figure S6: HPLC purity data of the NDI derivative. The analytical chromatogram of a NDI sample assessed by Method-2 has been reported. HPLC flux 1.4 ml/min.

Supplementary Table S1: Oligonucleotides used in this study

Application	Name	Sequence (5′→3′)
CD	RET FL	TTTTTGC**GGG**TA**GGGGC**G**GGGGC**G**GGGGC**G**GGGG**CGGTTTTT
	RET I-IV	TTTTTA**GGGGC**G**GGGGC**G**GGGGC**G**GGGG**CGGTTTTT
	RET II-V	TTTTTGC**GGG**TA**GGGGC**G**GGGGC**G**GGGG**CTTTTT
	RET FL Mut	TTTTTGC**GGG**TA**GGTGC**G**GGGGC**G**GGAGC**G**GGGG**CGGTTTTT
	RET I-IV Mut	TTTTTA**GGTGC**G**GGGGC**G**GGAGC**G**GGGG**CGGTTTTT
	RET II-V Mut	TTTTTGC**GGG**TA**GGTGC**G**GGGGC**G**GGAG**CTTTTT
	c-Myc	T**GGGGA**G**GGT**G**GGGGA**G**GGT**G**GGGG**AAGG
	c-Kit1	A**GGGA**G**GGC**GCT**GGG**AGGA**GGG**
Taq polymerase stop assay	RET Taq primer	GGCAAAAAGCAGCTGCTTATATGCAG
	RET FL Taq	TTTTT**GCGGGTA**G**GGGGC**G**GGGGC**G**GGGGC**G**GGGGG** CGGTTTTTCTGCATATAAGCAGCTGCTTTTTGCC
	RET I-IV Taq	TTTTTA**GGGGC**G**GGGGC**G**GGGGC**G**GGGGG**CGG TTTTTCTGCATATAAGCAGCTGCTTTTTGCC
	RET Mut FL Taq	TTTTTGC**GGG**TA**GGTGC**G**GGGGC**G**GGAGC**G**GGGGG**CGG TTTTTCTGCATATAAGCAGCTGCTTTTTGCC
	RET Mut I-IV Taq	TTTTTA**GGTGC**G**GGGGC**G**GGAGC**G**GGGGG**CGG TTTTTCTGCATATAAGCAGCTGCTTTTTGCC
	Control Taq	TTGTCGTTAAAGTCTGACTGCGAGCTCTCA GATCCTGCATATAAGCAGCTGCTTTTTGCC
Cloning of pGL3-RETmut	Forward primer	GTCAGGTACCGCCCCTTCCCGCACCCCACCCGCCTCCGGCCCCGC CTGGCCCACCCCTGGACCGCCCCCGC**T**CCGCCCCGC**A**CCTACCCG CTCC
	Reverse primer	TGACCAAGCTTGTTCCGGGGCACTCAGCGCT

G-tracts are shown in bold. Mutated bases are underlined.

Supplementary Table S2: Stabilization of the G4s of *RET*, *KIT1* and *MYC* promoters (4 µM) by the NDI (4 µM) in 10 mM KCl

Sequence	ΔTm (°C)
RET FL	1.3 ± 0.5
RET I-IV	1.2 ± 0.1
RET II-V	5.6 ± 0.4
c-Kit1	1.5 ± 0.1
c-Myc	3.6 ± 0.3