Electronic Supplementary Material (ESI) for Chemical Communications.

Supplementary Information

End-ligation can dramatically stabilize i-motifs at neutral pH

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Outline

1. Materials and Methods

Oligonucleotide synthesis and purification

Oligonucleotide synthesis was performed on an ABI 3400 DNA synthesizer (Applied Biosystems) at 1 μmol scale on Unylinker (ChemGenes) CPG solid support, unless sequences required a 3′-phosphate group for which 3′-phosphate-ON Icaa CPG (Chemgenes) was used. Thymidine (dT), deoxycytidine (N-acetyl) (dC), deoxyguanosine (N-ibu) (dG) and deoxyadenosine (N-Bz) (dA) phosphoramidites were used at 0.1 M concentration in acetonitrile and coupled for 200 s. araF-C was used at 0.1 M concentration and coupled for 600 s. Bis(2-cyanoethyl)-N,N-diisopropyl phosphoramidite (Chemgenes) was used to attach 5′-phosphate groups and was used at 0.1 M concentration and coupled for 1000s. Oligonucleotide deprotection and cleavage from the solid support were achieved using 30% aqueous ammonium hydroxide at 55°C for 16 hours. The sequences were purified by anion exchange HPLC on a Waters 1525 instrument using a Source 15Q Resin column (11.5 cm × 3 cm). The aqueous buffer system consisted of Solution A (25% acetonitrile, 15 mM sodium acetate) and Solution B (0.5 M lithium perchlorate, 25% acetonitrile, 15 mM sodium acetate) (solution A) at a flow rate of 10 mL/min. The gradient was 0−100% lithium perchlorate over 50 minutes at 60 °C. Under these conditions the desired peaks eluted at roughly 23-32 min. The purified oligonucleotides were desalted using Glen-pack desalt columns (Glen Research), and their masses were confirmed by high-resolution LC-MS.

Ligation with N-cyanoimidazole

Oligonucleotide solutions (50 μM; alone or with equimolar amounts of template) were annealed in 100 mM MES, 50 mM KCl (pH 5) slowly to room temperature and stored at

5°C overnight. While maintaining the sample at 5°C, an equal volume of freshly prepared 100 mM N-cyanoimidazole, 100 mM $MnCl_2$ in water was added. The samples were vortexed and allowed to react overnight at 5°C. Note that N-cyanoimidazole does not readily dissolve in water and requires sonication and vortexing. The oligonucleotides were precipitated with at least 5x volume of 2% lithium perchlorate in acetone on dry ice over at least 30 minutes. The samples were then centrifuged and the supernatant was discarded. Note that some N-cyanoimidazole might be part of the pellet; washing with cold acetone and centrifuging again helps dissolve the N-cyanoimidazole. The pellet was dried and dissolved in a 1:1 H_2O/formamide to prepare the sample for gel electrophoresis. The samples were heated at 95°C for one minute and allowed to cool for 2 minutes before loading into a 20% denaturing polyacrylamide gel. Samples were electrophoresed at 300 V for 1.5 hours, and the bands of the gels were then revealed by UV-shadowing. For preparative gels, the band containing the desired ligated product was cut from the gel into small pieces, soaked in 0.1 M sodium bicarbonate, and left shaking overnight at room temperature. The aqueous solution, now containing the oligonucleotides, was filtered and desalted using Glen-pak desalt columns. The masses of the ligation products were confirmed by high-resolution LC-MS.

Ligation with cyanogen bromide

50 μM oligonucleotide solutions (alone or with equimolar amounts of template) were annealed in 250 mM MES, 50 mM NaCl (pH 6) slowly to room temperature and stored at 5°C overnight. While maintaining the sample at 5°C, a 14% volume of freshly prepared 3.5 M CNBr, 300 mM $MgCl_2$ in water was added. The samples were vortexed and allowed to react for 5 minutes at 5°C. The oligonucleotides were precipitated with at least 5x

volume of 2% lithium perchlorate in acetone on dry ice over at least 30 minutes. The samples were then centrifuged and the supernatant was discarded. Note that some NCI might be part of the pellet; washing with cold acetone and centrifuging again helps dissolve the NCI. The pellet was dried and dissolved in a 1:1 H_2O/formamide to prepare the sample for gel electrophoresis. The samples were heated at 95°C for one minute and allowed to cool for 2 minutes before loading into a 20% denaturing polyacrylamide gel. Samples were electrophoresed at 300 V for 1.5 hours, and the bands of the gels were then revealed by UV-shadowing. For preparative gels, the band containing the desired ligated product was cut from the gel into small pieces, soaked in 0.1 M sodium bicarbonate, and left shaking overnight at room temperature. The aqueous solution, now containing the oligonucleotides, was filtered and desalted using Glen-pak desalt columns. The masses of the ligation products were confirmed by high-resolution LC-MS.

CD spectroscopy

Circular Dichroism (CD) studies were performed at 5 °C on a JASCO J-810 spectropolarimeter, equipped with a Peltier temperature controller. Samples consisted of 15 μM concentrations of oligonucleotide dissolved in 10 mM NaP_i buffer of desired pH. Samples were heated at 95°C and annealed slowly to room temperature over 3 hours and placed at 5°C overnight. The final pH of the samples was confirmed prior to any CD measurement. Samples were added to a 1 mm path length cuvette for measurement. Spectra were recorded from 350-230 nm at a scan rate of 100 nm/min and a response time of 2.0 s with three acquisitions recorded for each spectrum. The spectra were normalized by subtraction of the background scan with buffer. Data were smoothed using the Savitsky-Golay function within the JASCO graphing software.

UV-melting analysis

UV-based thermal denaturation experiments were performed on a Cary 100 UV-Vis spectrophotometer equipped with a Peltier temperature controller. 0.5 OD samples were prepared in 10 mM sodium phosphate buffer (pH 5 or 7). Samples were heated at 95°C and annealed slowly to room temperature over 3 hours and placed at 5°C overnight. Acquisitions were performed at 265 nm and in 1 mm path-length cuvettes. Absorbance values were acquired at a heating rate of 0.5 °C/min. Experiments for all samples were repeated at least three times, and only one dataset is represented in the figures of this study. Thermal stability (T_m) values were determined as the maxima in the first-derivative plots of the melting curves.

Non-denaturing PAGE

Oligonucleotide samples were analysed using native gels consisting of acrylamide/bis 19:1 (20%), 10 mM sodium phosphate pH 7, and 1x TBE (Tris-Borate-EDTA). The final gel mix solution was adjusted to pH 7 prior to casting.

Oligonucleotide samples (0.03 ODs) were heating to 95°C in 10 mM sodium phosphate pH 7, annealed slowly to room temperature over 3 hours and stored overnight at 5°C. They were mixed with 25% glycerol to attain a final glycerol concentration of approx. 11.5% before loading them in the gels.

After casting the gels and loading them with sample, they were run at 19 V/cm over 2 hours and 7°C, using 1x TBE pH 7 as running buffer. Gel results were visualized using Stains-All. Xylene cyanol and bromothymol blue dyes were used to monitor the progress of electrophoresis.

2. Supplementary Figures

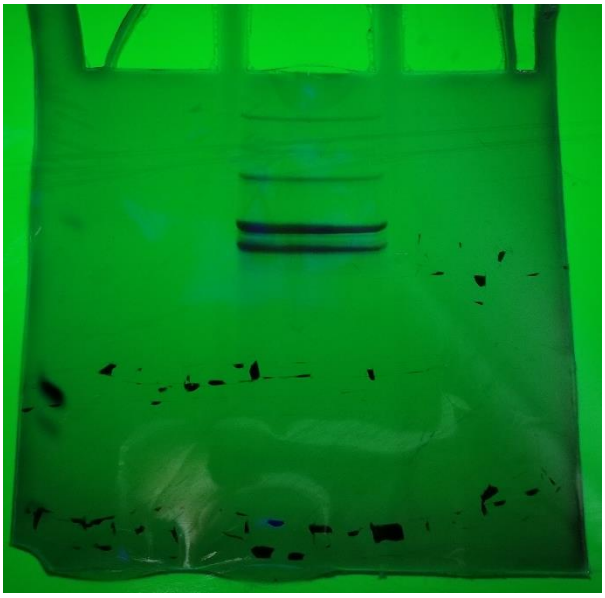

Figure S1. Uncropped, unprocessed denaturing polyacrylamide (20%) gel electrophoresis (PAGE) results, revealed by UV-shadowing, show the separation of Tel-2CC-ligated from Tel-2CC, as well as the formation of two lower-mobility dimeric side-products. The corresponding cropped and processed image is included in **Figure 1**.

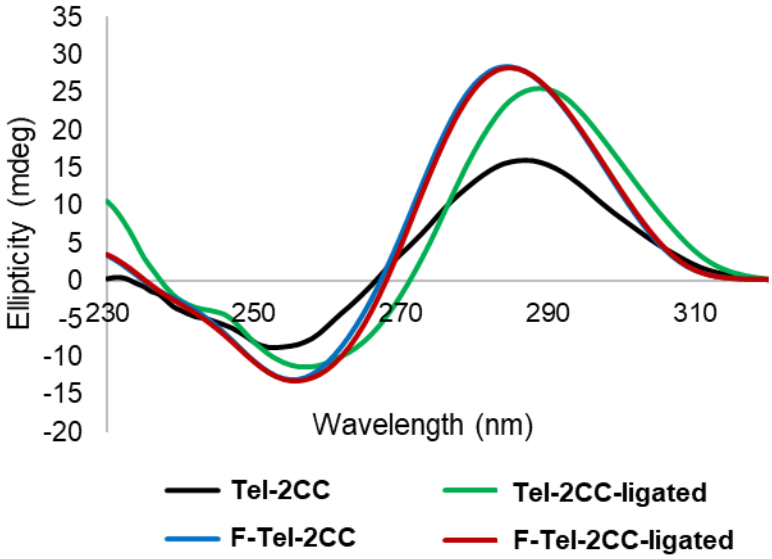

Figure S2. CD spectra at 5°C of Tel-2CC, F-Tel-2CC, and their ligated counterparts at pH 7. Samples are in 10 mM NaP_i buffer.

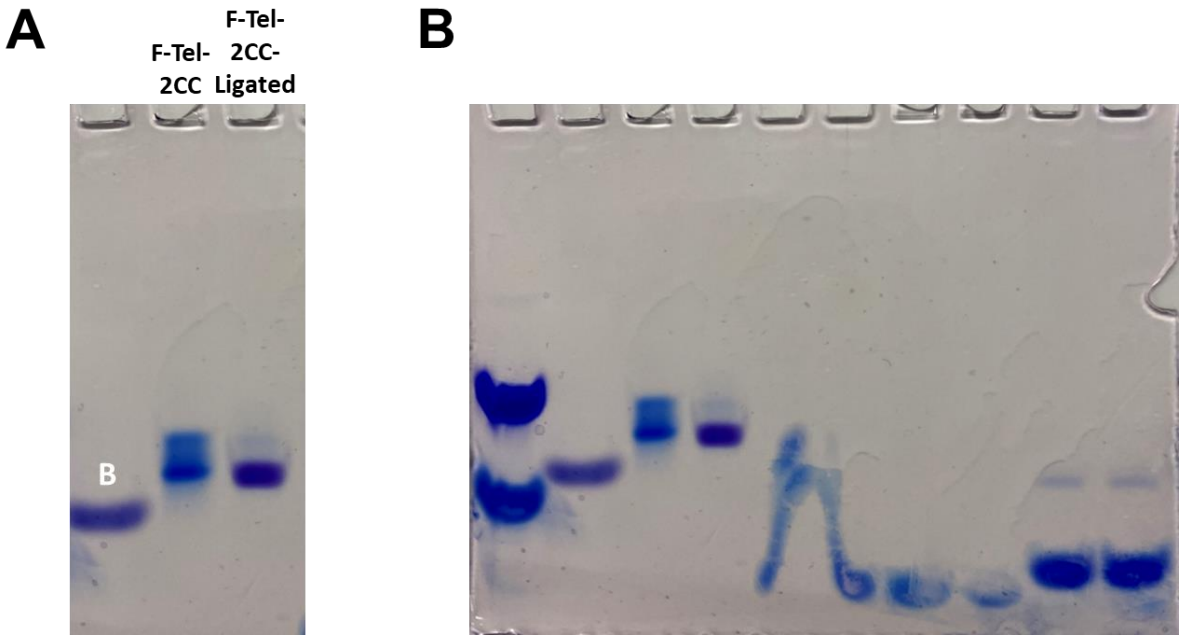

Figure S3. (**A**) Non-denaturing PAGE (20%) of ligated and unligated araF-C modified F-Tel-2CC. The bands have been stained with Stains-All. Band B represents bromothymol blue used to track the progress of electrophoresis. (**B**) Original uncropped gel image containing the lanes in (**A**) as well as other irrelevant lanes.

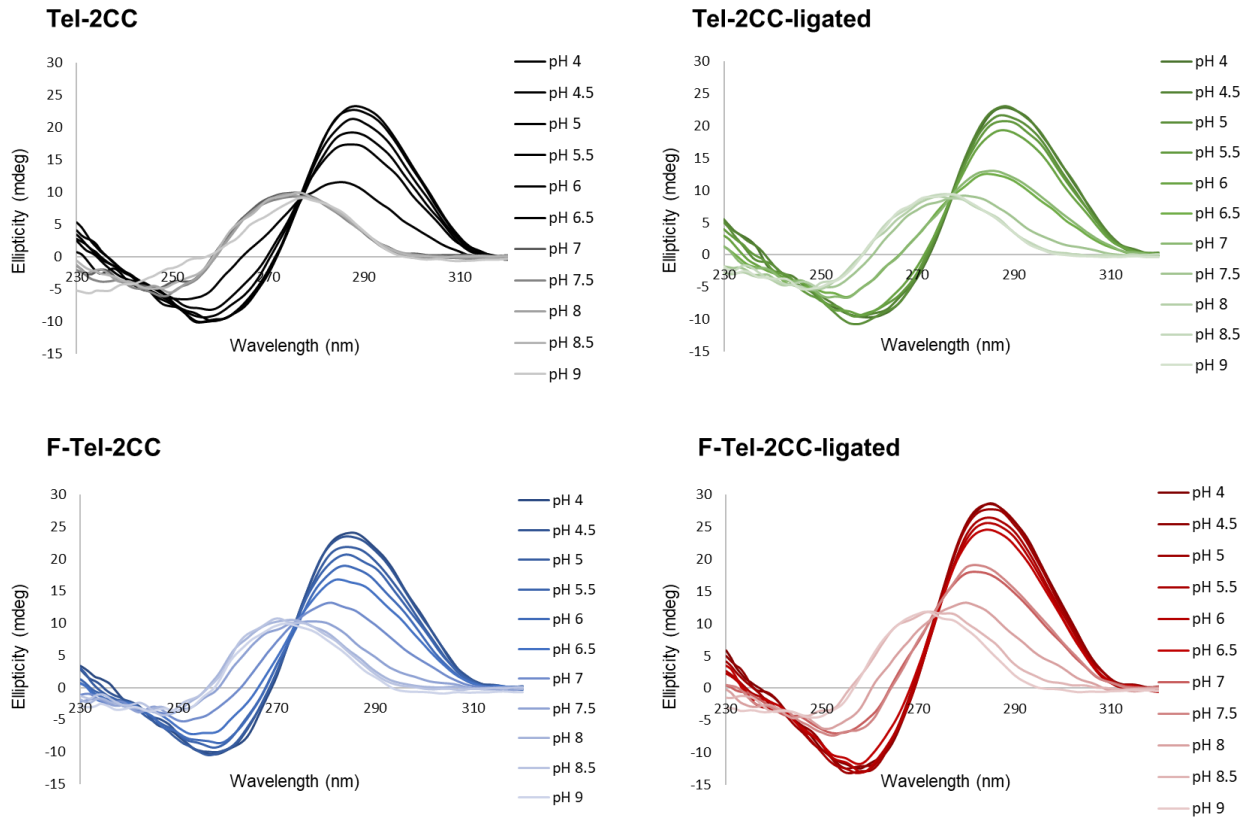

Figure S4. CD spectra showing the pH response at 5°C of Tel-2CC, F-Tel-2CC, and their ligated counterparts. Samples are in 10 mM NaP_i.

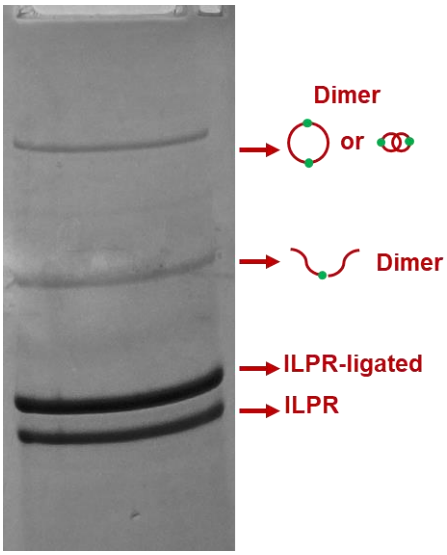

Figure S5. Denaturing polyacrylamide (20%) gel electrophoresis (PAGE) results, revealed by UV-shadowing, show the separation of ILPR-ligated from ILPR, as well as the formation of two lower-mobility dimeric side-products.

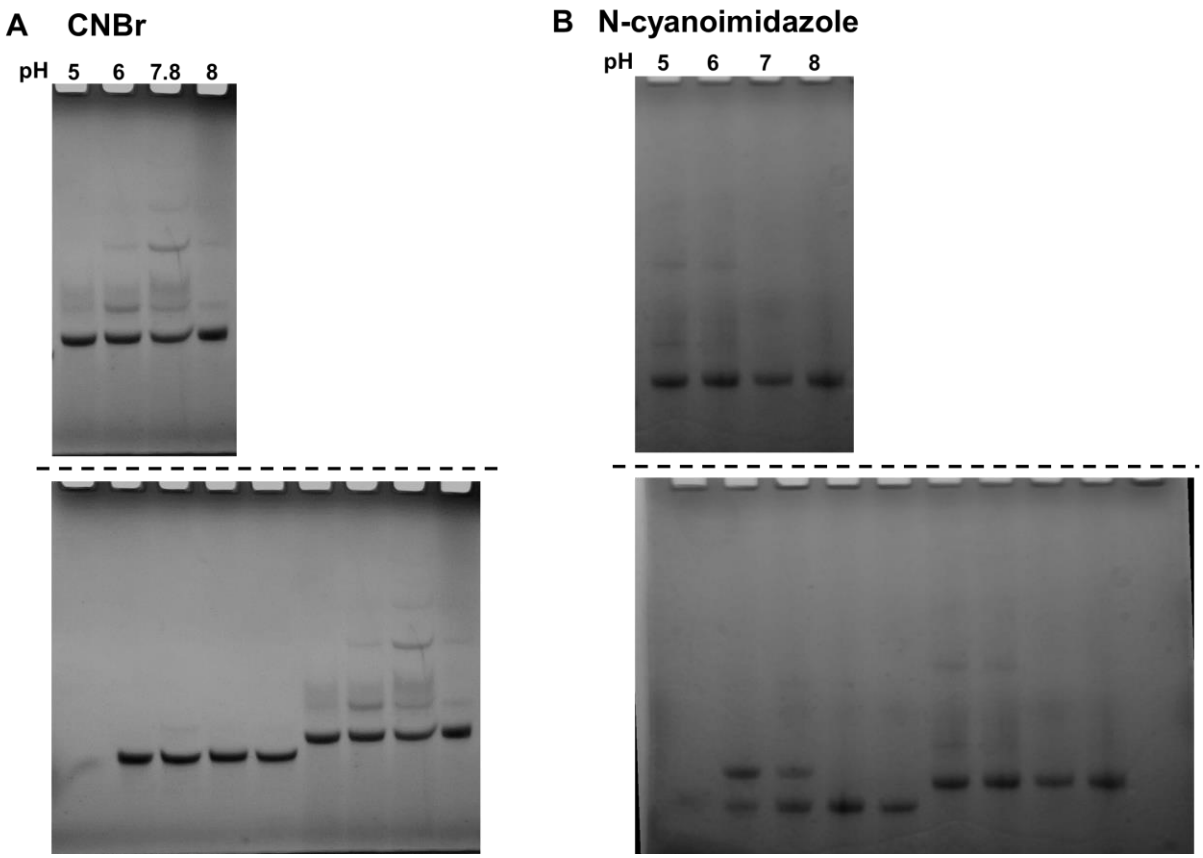

Figure S6. Ligation reactions attempted on KRASn-3p in the presence of 10-nt template, with CNBr (**A**, top) or N-cyanoimidazole (**B**, top) and at different pH. The CNBr reaction at pH 6 leads to the most ligation product. Images of corresponding uncropped gels including irrelevant lanes are included in (**A**, bottom) and (**B**, bottom), respectively.

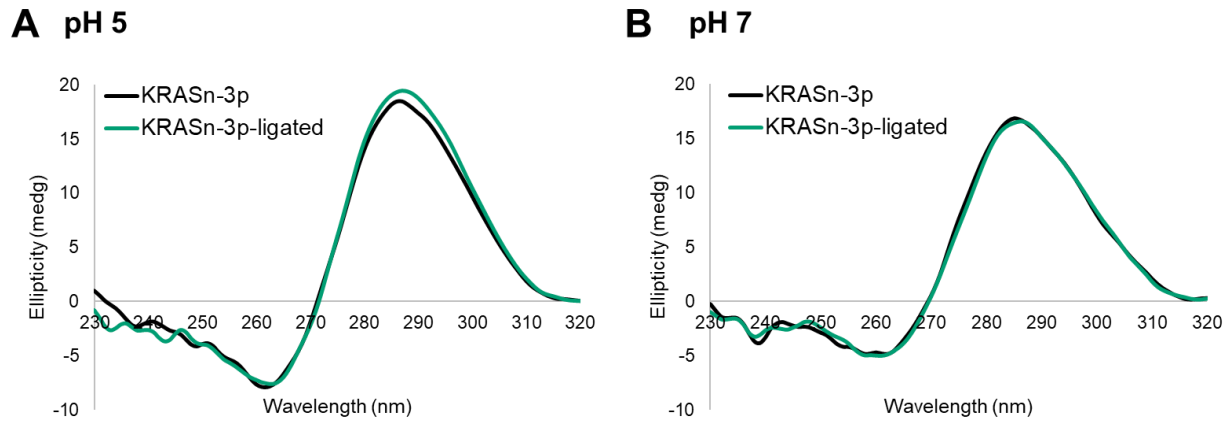

Figure S7. CD spectra at 5° of KRASn-3p and KRASn-3p-ligated in 10 mM NaPi at pH 5 (**A**) and pH 7 (**B**).

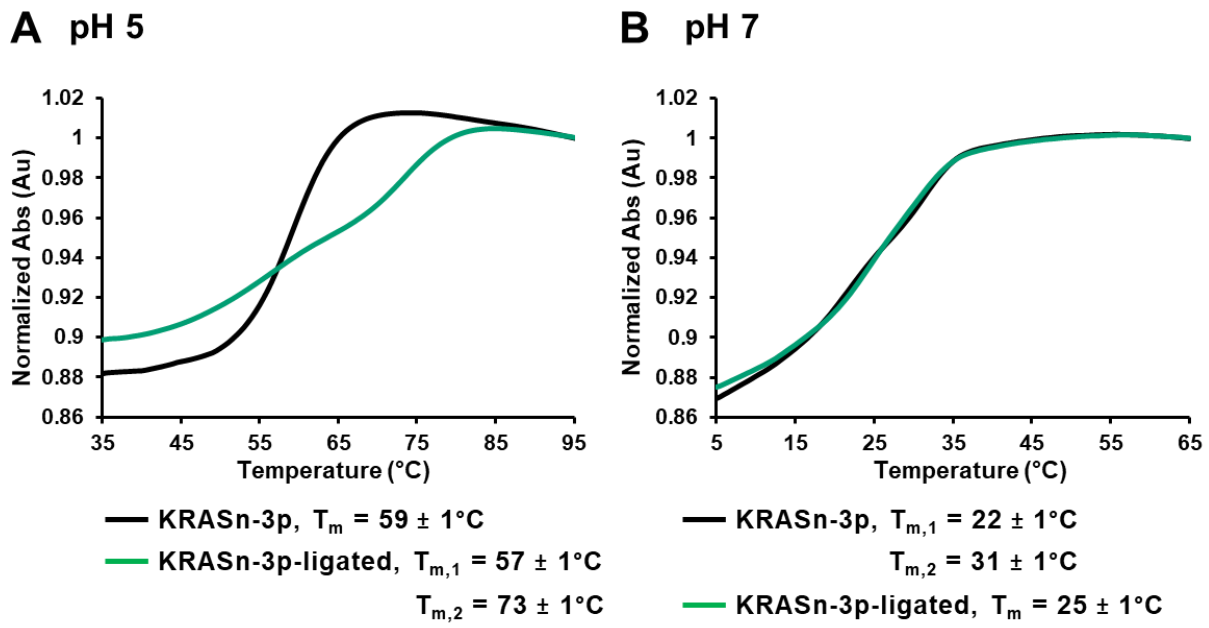

Figure S8. UV-melting curves of KRASn-3p and KRASn-3p-ligated at (**A**) pH 5 and (**B**) pH 7. Samples are in 10 mM NaP_i buffer.

Supplementary Tables

Table S1. i-Motif sequences used for ligation reactions, along with ligation strategy

Name	Sequence (5′-3′)*	Ligation Strategy
Tel-2CC	pCC(TA_2C_4)$_3$$TA_2$CC	C-tract
F-Tel-2CC	p**CC**(TA_2**C**$_4$)$_3$$TA_2$**CC**	C-tract
ILPR	CCTGTC_4ACAC_4TGTC_4ACACCp	C-tract
Telomeric-22	p$TC_3TAAC_3TAAC_3TAAC_3$	Loop construction
Telomeric-23	p$TC_3TAAC_3TAAC_3TAAC_3$T	Loop construction
Telomeric-24	pAA$TC_3TAAC_3TAAC_3TAAC_3$	Loop construction
C-Telomeric-5p	pCTAA$C_3TAAC_3TAAC_3$TAACC	C-tract
C-Telomeric-3p	CTAA$C_3TAAC_3TAAC_3$TAACCp	C-tract
KRASn-5p	p$TC_5TCTTC_3TCTTC_3ACACCGC_3$T	Loop+Template
KRASn-3p	$TC_5TCTTC_3TCTTC_3ACACCGC_3$Tp	Loop+Template

*p represents a terminal phosphate; **C** (bold C) represents araF-C

Table S2. Ligation reaction conditions used, along with the sequences tested

Conditions of ligation reaction	Sequences tested
N-cyanoimidazole, 100 mM MES, 50 mM KCl, 50 mM $MnCl_2$ pH 5	Telo-22, Telo-23, Telo-24, C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
N-cyanoimidazole, 100 mM MES, 50 mM KCl, 50 mM $MnCl_2$ pH 6	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
N-cyanoimidazole, 100 mM MES, 50 mM KCl, 50 mM $MnCl_2$ pH 7	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
N-cyanoimidazole, 100 mM MES, 50 mM KCl,50 mM $MnCl_2$ pH 8	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
CNBr, 250 mM MES, 50 mM NaCl, 20 mM $MgCl_2$ pH 5	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
CNBr, 250 mM MES, 50 mM NaCl, 20 mM $MgCl_2$ pH 6	Telo-22, C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
CNBr, 250 mM MES, 50 mM NaCl, 20 mM $MgCl_2$ pH 7.6	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p
CNBr, 250 mM MES, 50 mM NaCl, 20 mM $MgCl_2$ pH 8	C-telo-5p, C-telo-3p, KRASn-5p, KRASn-3p