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13. SUPPLEMENTARY NOTES

14. ABSTRACT
The source of mutagenesis for the establishment and maintenance of cancer is complex. However, defects in DNA repair genes in the double-strand break repair pathway are cancer predisposing. My lab has characterized a new potentially important source of double-strand breaks (DSBs) in human cells and are interested in characterizing which DNA repair genes act on this particular source of DNA damage. Selfish DNA accounts for 45% of the human genome. We have recently demonstrated that one particular selfish DNA, the L1 retrotransposon, creates DSBs via its endonuclease domain.
The important conclusions from the published work are that 1) the human retrotransposon L1 creates DSBs and 2) the DSB repair gene ATM is required for L1 retrotransposition.
To additionally characterize the roles of ATM and the ATM-related genes, BRCA1 and BRCA2, in L1-induced DSBs, we developed a new vector system to suppress their expression which is compatible with L1 assays. These constructs should also be of general utility.

15. SUBJECT TERMS
BRCA1, BRCA2, double-strand breaks, retrotransposition, LINE-1, ATM

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Introduction
The source of mutagenesis for the establishment and maintenance of cancer is complex. However, defects in DNA repair genes in the double-strand break repair pathway are cancer predisposing. My lab has characterized a new potentially important source of double-strand breaks (DSBs) in human cells and are interested in characterizing which DNA repair genes act on this particular source of DNA damage.

Prior to the funding of this grant we had unpublished data demonstrating that the human mobile element, L1, is a potent inducer of DSBs in cells (1). We also had preliminary data suggesting the DSB repair gene ATM is involved in the L1 retrotransposition process. ATM is known to interact with the breast cancer oncogenes, BRCA1 and BRCA2, in DSB repair. We wished to establish a role for BRCA1/2 directly in responding to L1 activity. The scope of the grant proposal was to use molecular and immunostaining to demonstrate activation of BRCA1/2 by L1 and an effect of BRCA1/2 deficiency on L1 activity.

Body
It is worth noting that the term of this one year grant began one month prior to the evacuation of New Orleans due to Katrina. This complication severely impacted the timeframe to conduct the proposed research projects. The important conclusions from the published work are that 1) the human retrotransposon creates DSBs and 2) the DSB repair gene ATM is required for L1 retrotransposition. 3) We have also developed a useful modification of shRNAs for inhibiting specific gene expression for the purposes of the proposed studies.

Task 1. Determine whether L1 induces activation of BRCA1
The antibodies to determine whether S1387 on BRCA1 is phosphorylated were purchased. We are currently determining the conditions for the antibody in Western blotting using ionizing radiation as a positive control. Antibodies for phospho-p53 and phospho-Chk2 have also been purchased and are additionally being optimized in Western blotting.

Task 2. Characterize the localization of BRCA1 and BRCA2 and related proteins into foci after L1 expression. The antibodies for detection of BRCA1 and BRCA2 have been purchased. We are currently determining the conditions for the antibodies for immunofluorescence using ionizing radiation as a positive control.

Task 3. Determine whether BRCA1 and BRCA2 play a role in the retrotransposition of L1.
The majority of the work related to this task revolved around making transient and stable RNAi knockdown vectors targeting BRCA1/2. We have demonstrated that standard shRNA, RNAi vectors are incompatible with the L1 assay and L1 toxicity assays (2). However, we also developed a new vector technology that overcomes these limitations (2) which we planned to use for this grant. The revision of the accepted paper and further development of these vectors was performed during the term of this grant. Using standard hairpin optimization techniques, we developed BRCA1, BRCA2, and ATM targeting hairpins using these modifications in our new vector system. However, we found using a test target (EGFP) that certain variations on hairpin design using our modified vector

system lost activity. While the specifics of why the new designs don't work are not known, we have recently finished creating new targeting hairpins using the original, canonical design. We've made BRCA1, BRCA2, and ATM targeting hairpins that have shown efficacy in publications from other labs. We are currently using Western blotting techniques to determine the actual knockdown in HeLa cells.

The final task was to perform assays using the known BRCA1-deficient cell line HCC1937. We obtained this cell line and began preliminary tests to optimize the various colony plating assays my lab utilizes in other cell lines. We found that this cell line under our standard assay conditions was not efficiently killed by the selection reagent, gneticin. All of my lab's L1 assay vectors use geneticin resistance. We plan on testing different selection vectors that can be used for the L1 toxicity assays and developing different resistance markers for our L1 assay vectors.

Key Research Accomplishments
- The key role of ATM in mobile element insertions has been solidified and submitted as a manuscript, including a demonstration that ATM is not needed for the DSB activity of L1, but is needed for insertion (data appended).
- We have created a shRNA vector system capable of medium –term (weeks) knockdown that avoids some of the side toxicities associated with commercial shRNA vector systems.

Reportable Outcomes and Abstracts
1. Submission for publication of results: The further development of new vector systems for RNAi which are compatible with L1 assays. Creation of BRCA1, BRCA2, and ATM targeting hairpins using this new vector system. "Stable gene suppression with BC1-linked hairpins." submitted to Biotechniques

2. In preparation for submission for publication of results demonstrating that ATM is required for L1 retrotransposition. " ATM plays an important role in LINE-1 Retrotransposition." (to be submitted to Current Biology)

3. American Society for Human Genetics Annual Meeting: ATM Satellite Meeting, New Orleans, LA 10/2006 and American Society for Human Genetics Annual Meeting, New Orleans, LA 10/2006 ! "Role of ATM During L1 Retrotransposition"

4. 4th Annual International Conference on Transposition and Animal Biotechnology, Minneapolis, MN, 6/2006 "SINEs as carriers of RNA hairpins for nontoxic RNA interference"

Conclusions
This work has demonstrated that an important source of endogenous DSB activity could be highly active and mutagenic in human cells. This activity of the L1 retrotransposon has been previously underappreciated relative to its insertional mutagenesis. Extension of this result will be to understand the actual activity and mutagenesis contribution of L1 in human somatic cells. The second

major conclusion is the first demonstration that a DNA repair gene (ATM) is required for L1 retrotransposition. We specifically demonstrate that ATM is not required for L1 to create DSBs, suggesting that other repair proteins can important for processing L1-induced DSBs. The evaluation of additional DNA repair genes, like BRCA1 and BRCA2, should be a fruitful area of research for understanding L1 retrotransposition as well as the mutagenic effects of DSB processing in DNA repair mutants.

In regards to human health, and breast cancer in particular, this work suggests that targeting L1 endonuclease activity may help limit cancer development and/orprogression.

References

S.L. Gasior, T. P. Wakeman, B. Xu, P. L. Deininger. 2006. The human LINE-1 Retrotransposon Creates DNA Double-Strand Breaks. J. Mol. Biol. 357: 1383-93.

S.L. Gasior, M. Palmisano, P. L. Deininger. 2006. Alu-linked hairpins efficiently mediate RNA interference with less toxicity than H1-expressed shRNAs. Anal Biochem. 349: 41-8.

Appendices:
1) Manuscript and Meeting Abstracts.
2) Data showing DSBs from L1 in ATM- cells
3) Submitted manuscript

1. In preparation for submission for publication of results demonstrating that ATM is required for L1 retrotransposition. " ATM plays an important role in LINE-1 Retrotransposition." (to be submitted to Current Biology)

ABSTRACT:
Long INterspersed Element-1 (L1) is an autonomous retroelement that is active in the human genome. Even though L1-promoted retroposition is responsible for approximately 28% of the sequence, little is known about the interaction of host factors with L1 or other non-Long Terminal Repeat (non-LTR) retrotransposon integration intermediates. The proposed mechanism of insertion for non-LTR retrotransposons requires cleavage of both strands of genomic DNA, suggesting a double-strand break (DSB) is an intermediate during integration. Therefore, we sought to characterize the role ATM (Ataxia-Telangiectasia Mutated) might play in L1 retrotransposition, given ATM's role in mediating DSB repair in response to radiation. Inhibition of ATM with caffeine, wortmannin, or a dominant negative allele all negatively impact L1 retrotransposition. Cell lines derived from AT patients poorly support L1 retrotransposition, but the defect is complemented by wild-type ATM expression. These data suggest that ATM is required for processing L1 retrotransposition intermediates and endogenous L1 activity may underlie some aspects of AT, particularly cancer predisposition.

2. American Society for Human Genetics Annual Meeting: ATM Satellite Meeting, New Orleans, LA 10/2006 and American Society for Human Genetics Annual Meeting, New Orleans, LA 10/2006 ! "Role of ATM During L1 Retrotransposition"

ABSTRACT:
Mobile elements represent a source of insertional mutagenesis in all genomes studied to date. The human L1 belongs to the non-Long Terminal Repeat class of retrotransposons and is the only known active autonomous element in the human genome. L1 encodes an APE-like endonuclease and a reverse transcriptase that enable the L1 RNA to initiate its integration into the genome, a process termed Target Primed Reverse Transcription (TPRT). TPRT suggests that a double-strand break (DSB) would be a potential intermediate, and we have demonstrated previously that overexpression of L1.3 induces gamma-H2AX foci and COMET tails under neutral conditions. Both are hallmarks of DSBs. To further understand the role of cellular DNA repair proteins in response to L1 DSB formation and integration, we are characterizing whether ATM is activated and/or required during L1 TPRT. ATM is an important kinase for the repair of DSBs. After expression of L1.3, ATM autophosphorylates and ATM-P localizes on chromatin as foci similar to how ATM responds to ionizing radiation. We have also tested the requirement for ATM in a colony plating assay in which colonies represent a cell with an L1 integration. Treatment of cells with wortmannin or caffeine, two molecules that inhibit ATM kinase, lowers the frequency of colonies. Further analyses with an ATM dominant negative allele and AT-deficient cells suggest L1 integration specifically requires ATM. These results impart an important new understanding for sources of DNA damage that leads to genetic

instability in humans. Because L1 expression is elevated in many cancer cells, it may be a major contributor of DSBs, which in turn promote chromosomal rearrangements. In cells deficient in DSB repair, there may be additional consequences for genetic instability due to L1 activity.

3. 4th Annual International Conference on Transposition and Animal Biotechnology, Minneapolis, MN, 6/2006 "SINEs as carriers of RNA hairpins for nontoxic RNA interference"

ABSTRACT:
RNA interference is a powerful tool to specifically reduce the expression of target genes in a wide variety of model organisms. The active RNA intermediate is a short RNA with sequence complementary to the target mRNA—the guide strand. A series of processing steps on a pol II RNA with a hairpin structure leads to short duplexes, one of which is the guide strand. Pol III-promoter hairpin expression systems have been shown to enter the RNAi pathway and also mediate silencing and are a popular technique for knockdown in mammalian cells. Recent work has shown, however, that these expression systems can have off-target side effects including cytotoxicity and induction of the interferon response. Some lentiviral hairpin expression constructs have been difficult to use for generation of transgenic mice. We demonstrate that expressing RNA hairpins in the same transcript as either Alu or the ID portion of mouse BC1 mediate silencing of target transgenes without toxicity. Alu and ID are Short INterspersed Elements (SINEs) in the primate and rodent genomes that are still active and produce new insertions. Both elements are retroposons that use their host genome LINE-1 retrotransposons for mobilization. The ability of Alu and ID to act as non toxic carriers for RNAi-mediating hairpins may be due to cellular or element adaptations that avoid impacting RNA metabolism. Our most recent results demonstrate that both Alu and ID with hairpins expressed from episomal vectors can knockdown target gene expression (EGFP) in HeLa cells for up to two months. These results highlight the usage of retroelements in mediating RNAi and potentially provide a more robust technique that minimizes the well-documented sideeffects of standard RNAi expression systems.
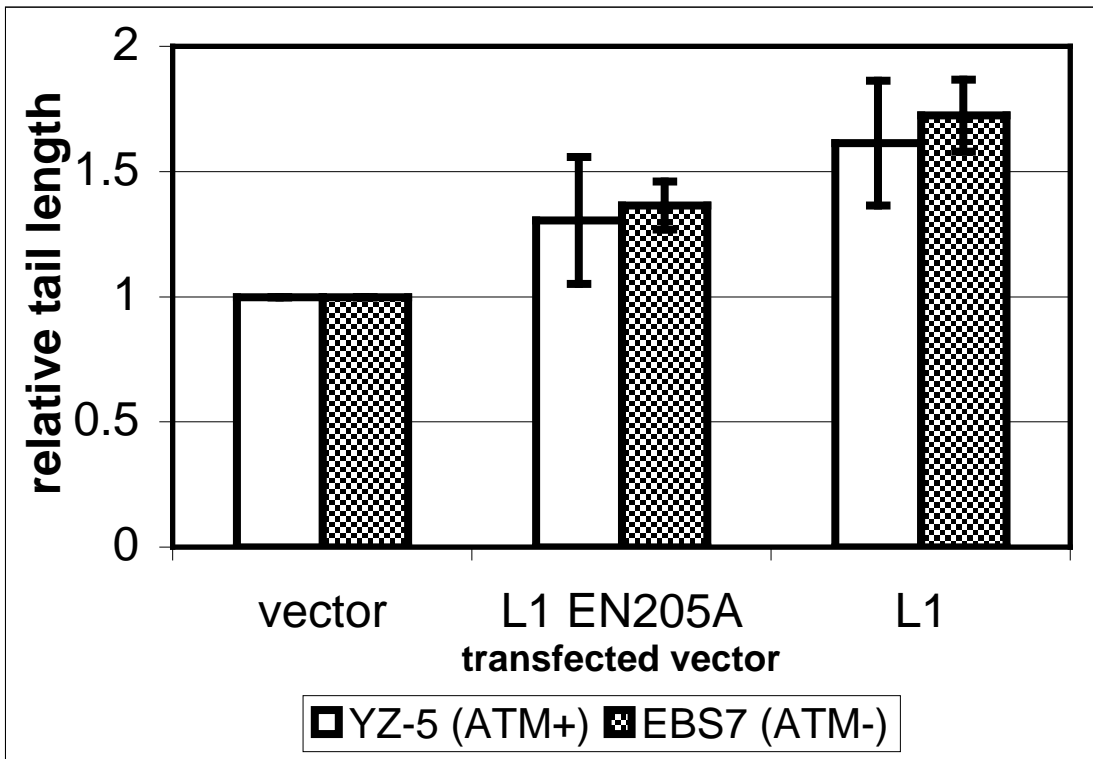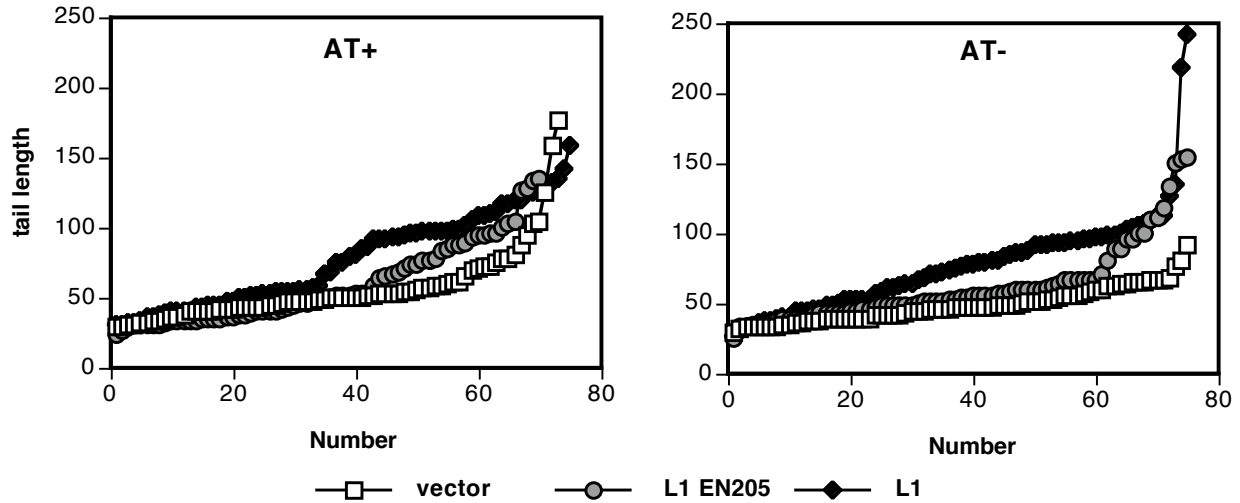
DSB formation in AT- cells. The AT derived cell lines YZ-5 (ATM complemented) and EBS-7 (vector control) were transfected with L1 expression constructs (pBud_L1_optORF1_optsynORF2, EN205A mutant) or vector control (pBud) and harvested 24 hours later and subjected to neutral COMET assay. Sample experiment of COMET tail lengths in the whole population of cells. Quantiation of two replicates of neutral COMET assays relative to empty vector tail length.

Manuscript Number:

Title: Stable gene suppression with BC1-linked hairpins

Article Type: Research Report

Corresponding Author: Postdoctoral Fellow Stephen Gasior, Ph.D.

Corresponding Author's Institution: Tulane University

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Order of Authors: Stephen L Gasior; Yi-Chuan Luk; Zach Faber; Prescott L Deininger

Abstract: RNA interference has become a powerful tool for the specific inhibition of genes in mammalian cells. Previously, Alu-linked hairpins were demonstrated to transiently mediate RNA interference without associated toxicity in contrast to hairpins expressed from upstream RNA pol III promoters. To expand upon those results, we now demonstrate that hairpins attached to the ID portion of the rat neuronal RNA gene BC1 also demonstrate transient RNA interference with less toxicity than pol III-expressed hairpins. ID is a rodent-specific SINE. This result further corroborates the ability of repetitive element RNAs to act as neutral carriers of RNA interference-mediating hairpins. In addition, we demonstrate that BC1(ID) expression constructs mediate long-term suppression of EGFP in HeLa cells over the course of six months. Interestingly, Alu constructs generate robust but temporary target suppression demonstrating a de novo silencing of Alu expression. These vectors thus provide new tools for gene suppression with novel and potentially useful properties. The observed silencing of Alu expression in vivo also provides a new technique for understanding the regulation of SINE expression.

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Dear Mr. McCormick,

We are submitting the following Research Report manuscript "Stable gene suppression with BC1-linked hairpins." The results therein contain several novel observations that are relevant to the molecular biology technique of DNA-directed RNA interference. One novel result is the demonstration of stable target gene suppression by a hairpin carried by a portion of the rat BC1 gene. This portion corresponds to the ID family of repetitive elements. This type of expression construct may be favorable to use compared to standard hairpin constructs because we demonstrate less cellular toxicity by the BC1 constructs. This result is comparable to our previous publication demonstrating that the human Alu element is also an effective carrier of hairpins for RNAi and demonstrates less toxicity. In this manuscript we demonstrate that stably selected Alu hairpin constructs are rapidly silenced. This potentially provides a useful feature for certain applications of RNAi. In summary, we demonstrate a useful system for DNA directed RNAi with high efficacy and less side-effects for long-term studies.

S.L.G. is responsible for the overall design of the experiments, writing the manuscript, and experiments represented in Figures 2-4. Y-C. L. is responsible for experiments represented in Figure 1. Z. F. is responsible for most of the vector constructions and unreported preliminary results. P.L.D. provided valuable input in experimental design and revisions to the manuscript.

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Sincerely,

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Stable gene suppression with BC1-linked hairpins

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Running Title: Stable SeshRNAi

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Abstract

RNA interference has become a powerful tool for the specific inhibition of genes in mammalian cells. Previously, Alu-linked hairpins were demonstrated to transiently mediate RNA interference without associated toxicity in contrast to hairpins expressed from upstream RNA pol III promoters. To expand upon those results, we now demonstrate that hairpins attached to the ID portion of the rat neuronal RNA gene BC1 also demonstrate transient RNA interference with less toxicity than pol III-expressed hairpins. ID is a rodent-specific SINE. This result further corroborates the ability of repetitive element RNAs to act as neutral carriers of RNA interference-mediating hairpins. In addition, we demonstrate that BC1(ID) expression constructs mediate long-term suppression of EGFP in HeLa cells over the course of six months. Interestingly, Alu constructs generate robust but temporary target suppression demonstrating a *de novo* silencing of Alu expression. These vectors thus provide new tools for gene suppression with novel and potentially useful properties. The observed silencing of Alu expression *in vivo* also provides a new technique for understanding the regulation of SINE expression.

Key Words: RNA interference, Alu, SINE, shRNA, SeshRNA, retroelement, BC1, ID

Abbreviations:

EGFP	-Enhanced Green Fluorescent Protein
GFP	-EGFP targeting hairpin
Luc	-firefly luciferase targeting hairpin
miR	-microRNA
$G418^r$	-G418 resistance
RNAi	-RNA interference
SeshRNA	-SINE Enhanced Short Hairpin RNA
siRNA	-small interfering RNA
shRNA	-short hairpin RNA
UAS	-upstream activating sequence

Introductory Statement

RNA interference (RNAi) is a cellular system that controls gene expression by targeting specific mRNAs for destruction [1;2]. Short (~19-25 nts) RNAs, which are complementary to the target, initiate degradation of the mRNA, and these short interfering RNAs (siRNAs) can be generated artificially by vector systems [3]. The first generation of these vectors expressed RNA molecules with palindromic sequences that fold into short hairpin RNAs (shRNAs) using external pol III promoters like H1 [4], 7SK [5], or U6 [6-9]. These shRNAs are suitable substrates for Dicer, which cleaves them to siRNA that then mediate target degradation [10]. All of these expression constructs have been incorporated into stable/selectable viral and nonviral vectors [4;11]. While all of the above mentioned constructs have been used successfully for many experiments, several groups have reported side-effects: interferon induction [12-14], negative selection and toxicity in tissue culture [15-18] and toxicity in transgenic mice [13;18]. The degree of hairpin expression is proportional to the negative selection [16]. Initially, the thought was that the interferon response was the cause of toxicity; however, recent results suggest that titration of the miR biogenesis pathway may be the mechanism of toxicity [18-20]. Thus, issues and concerns remain regarding the use of these vector systems for clinical applications and more work needs to be done to understand the biogenesis and side-effects of shRNA molecules.

DNA directed RNA interference does not require the generation of hairpin only transcripts ("naked" hairpins) to ultimately make the effector siRNAs. Several other expression systems have been developed in which the hairpin is linked or incorporated into a larger RNA. The apparently exclusive mechanism of endogenous posttranscriptional gene silencing in mammalian cells is via translational inhibition [21]. Though some protein components are distinct from RNA degredation, an expressed palindromic sequence is still the precursor substrate

(micro-RNA or miR) to generate the silencing RNA duplex. The observation that many mammalian miRs exist within pol II transcribed precursor mRNAs with conserved RNA secondary structures [22] led to the development of additional vector systems utilizing pol II promoters [23-25]. Internal pol III promoters to drive hairpin expression have also been shown to mediate RNAi. The partial tRNA for valine and the tRNA for lysine have been demonstrated to act as effective carriers for shRNAs and induces substantial RNAi [26;27]. A viral RNA has also been shown to drive RNAi with the incorporation of a targeting palindrome [12]. The mouse neuronal RNA gene BC1 has been shown to mediate RNA interference in neuronal cells [28]. However, not all linked pol III genes work to mediate RNAi [29;30]. We previously demonstrated that a hairpin expressed from a human repetitive element, the Alu SINE, overcame toxicity issues while still mediating robust RNAi to transgenes [17]. We utilized the constitutive 7SL upstream activation sequence (UAS) to drive high levels of Alu expression in HeLa cells. We termed this combination of a SINE to an RNA hairpin SINE-enhanced short hairpin RNA (SeshRNA). Another group has also demonstrated that Alu-linked hairpins can drive RNA interference to transfected transgenes [31]. This latter report utilized an Alu-hairpin expression cassette that was directly derived from the human genome as well as expressed from the genomic locus suggesting that SeshRNAi may be an endogenous pathway of silencing.

To test whether this observation is extensible to other repeats, we now characterize the ID portion of rodent BC1 RNA as a carrier for hairpins to mediate RNA interference as well as test for toxicity in HeLa cells. In addition we now report the ability of Alu- and BC1(ID)-linked hairpins to function as stable gene suppressors when expressed from a selectable vector.

Materials and Methods

SeshRNA vector constructs

BC1(ID) with the 7SL upstream activating sequence (UAS) was used in previously

described plasmids [32] and were moved with a PCR amplification and cloning to the pSuper-adap

vector as performed for 7SL-Alu vectors [17]. Forward primer, Eco_Alu_F:

GGAATTCCCTCCAGACCGCCCAGTGTGGGTG reverse primer, Bgl_BC1_R:

GTGCCAGATCTCTTGTTTTTTTTTGGTC

DNA duplexes containing palindromic sequences were ordered from Integrated DNA

Technologies (Coralville, IA) and cloned into *Bgl* II and *Hin*d III sites of the 7SL_BC1(ID)

cloning cassette (Genbank Accession number EU088134) in the pSuper backbone. Luciferase

and the standard EGFP targeting hairpins and transcription terminators (-term) were used as

described previously [17].

All annealing oligonucletides for targeting EGFP are listed in Table 1.

To create selectable episomal vectors for SeshRNA expression, the pCEP4 vector was

used (Invitrogen, Carlsbad, CA). We utilized an in-house polylinker modified vector, pCEP4A.

Appropriate electrophoresed bands from pSuper_BC1(ID)+/-hairpins digested with *Bam*H I and

Kpn I were isolated and ligated into the pCEP4A backbone digested with *Bgl* II and Kpn I. This

Bgl II digest additionally eliminated the CMV promoter of the pCEP4 vector.

Transfer of the 7SL-AluA cassette (Genbank Accession number EU092257) +/- hairpins

from pSuper to pCEP4A followed the same strategy as BC1(ID). To create the

pCEP4_7SL_AluAA constructs the 2ndA oligoes were annealed and ligated into

pCEP4A_7SL_AluA digested with *Hin*dIII and *Xba*I. 7SL_AluAA cloning cassette has Genbank

Accession number EU092258. A new primer for reverse sequencing was also developed

pCEP_AluAA_R: AGACTGCAAAGTCTGCTCCAGGAT.

Transfections and EGFP FACS

Colony plating assays and transient transfections for subsequent EGFP analysis have

been described previously [17].

For establishment of stable SeshRNA expression cell lines, 100,000 HeLa cells were

seeded into each well of a six well plate or into T25s for transfection the following day with 0.5

µg of DNA per well or plate. 5 µL of Lipofectamine2000 was used per plate. Cells were exposed

to DNA/Lipofectamine complexes for approximately 24 hours and then underwent selection in

120 µg/mL of hygromycin (Invitrogen).

To prepare stable SeshRNA cells for FACS analysis, cells were trypsinized and a fraction

was centrifuged at 1200 rpm. Cells were washed with 1 mL PBS and resuspended in 0.5-2.0 mL

PBS and kept on ice until analysis with a Becton Dickinson LSR II (Franklin Lakes, NJ).

Analysis rate was set for each sample to 400-1000 cells/second and at least 10,000 events were

scored per sample.

Clonal EGFP+ HeLa

HeLa cells (ATCC Manassas, VA) were cultured in EMEM plus nonessential amino

acids and sodium pyruvate supplemented with 10% FBS (Gibco/Invitrogen Carlsbad, CA). A

distinct population of EGFP+ (greenish) cells had been established after transfection and

selection for pBB14 which contains the EGFP expression gene [17]. This distinct population

underwent a second round of FACS selection (Becton Dickinson, FACSAria) and growth in selection media (greenish 2). After 3 Months of passage, Geneticin selection was removed and cells passaged for another 4 months. The cells were serially diluted in 96 well plates and 14 isolates from the last dilution giving + wells were analyzed after 3 weeks of growth in nonselective media. One isolate (H) was utilized for further analysis. Clonal isolates were passaged without selection.

Results

RNAi and Minimal Toxicity by BC1(ID)

To test the efficacy and toxicity of transiently transfected BC1(ID) hairpins, the ID portion of BC1 was cloned into the same vector as used previously for Alu [17]. We chose to use only the ID portion of BC1 because it represents the retroposition competent portion of the BC1 gene leading to the ID family of repeats, a rodent-specific SINE [33]. Notably, this portion corresponds to the tRNA portion of BC1 from which BC1 evolved [34]. While other studies have utilized this portion of BC1 with its endogenous upstream activating sequences for RNAi [28], our cloning placed the rat BC1(ID) downstream of the human 7SL UAS. This combination has been shown to drive high levels of expression in both mouse and human cell lines and the whole mouse [32].

Previously we demonstrated transient knockdown of the EGFP transgene in HeLa cells using Alu-linked hairpins. We refined our expression model with a second FACS sort for the EGFP+ population (M&M). This created a population of EGFP expression in about 70% of the viable population with distinct separation from the EGFP- cells (data not shown). After transfection of this cell line with an EGFP targeting hairpin cloned into pSuper-BC1(ID), we saw

significant suppression of EGFP expression within 4 days (26% EGFP+), approximately

equivalent to similar Alu-GFP vectors (AluAA-GFP and AluAA-GFP2T) (Figure 1a). The

pSuper-BC1(ID) vector that contains a terminator in the cloning site demonstrated no reduction

of EGFP+ cells.

To test for abrogation of the toxicity side effect by BC1-linked hairpins, we cotransfected

a G418^r expression vector and assayed colony formation. Similar to our previous observation,

BC1(ID)-hairpin vectors showed little toxicity relative to H1-expressed hairpins (Figure 1b).

Both the BC1(ID) empty vector and BC1(ID)-term vectors showed slight toxicity relative to the

pSuper-term vector (about 25%). This compares to about 80% toxicity for the pSuper-adap and

pSuper-GFP vectors. BC1(ID) still abrogates the toxicity of the GFP hairpin as demonstrated by

the unappreciable difference between pSuper-BC1(ID)-term and pSuper-BC1(ID)-GFP. These

results demonstrate that BC1 under the influence of the human 7SL UAS, is capable of target

gene suppression in a transient assay with modest associated toxicity.

Stable knockdown with BC1(ID)

To determine whether BC1(ID) or Alu-linked hairpins could mediate long term

suppression under selection, the expression cassettes were cloned into an episomal vector

containing the hygromycin resistance gene (pCEP). As controls, the H1-hairpin expressing

portion of pSuper vectors were also cloned into the same sites. We noted that our 2x refined

EGFP+ cells had a negative population and this population grew over time. Thus, to make sure

negative selection or silencing of EGFP was not occurring over a long-term analysis independent

of RNAi, we isolated clones of the EGFP expressing pool via serial dilution and a stable EGFP+

cell line clone (G2H) was utilized. This clone demonstrated a relatively tight population of cells

that was 99% EGFP+ as compared to the previous nonclonal population (Figure 2a).

The initial analyses (3 weeks posttransfection) of pCEP_BC1(ID)-GFP hairpin expression vector demonstrated suppression of EGFP (Figure 2b) equivalent to the H1-GFP hairpin. Control vectors with hairpins targeting firefly luciferase (luc) demonstrated no EGFP knockdown (Figure 2b). EGFP expression was then analyzed at 1 month intervals from the date of transfection. Over the course of 6 months, both the H1-GFP and BC1(ID)-GFP maintained EGFP knockdown approximately equivalent to negative cells (Figure 2c). The terminal 2 months of BC1(ID)-GFP did show a subset of cells with intermediate levels of EGFP expression in contrast to H1-GFP which stayed completely negative (Figure 2d). This and an additional time course experiment demonstrated continued suppression with H1-GFP and BC1(ID)-GFP over the course of 6 months (Figure 2e).

Unstable knockdown with Alu

The initial analyses of all pCEP_AluA-GFP hairpin expression vectors demonstrated suppression of EGFP (Figure 3a) equivalent to 7SL_BC1(ID)-GFP and H1-GFP hairpins. In contrast, over time the AluA-GFP clones showed a loss of EGFP suppression such that by 4 months, the percentage of EGFP+ cells was largely the same as control cells (Figure 3bc). Several repeats of just the AluA-GFP constructs showed that, by 50-75 days, at least 50% of cells were EGFP+ (Figure 3d).

Common hairpin optimizations abrogate stable Alu RNAi.

The design of duplexes for creating hairpin expressing vectors is generally under several design constraints. First, optimal transcription start sites need to be included as well as

transcription terminator sequences [4-9;12]. Incorporation of hairpins into Alu and BC1(ID) transcripts allows one to potentially bypass these requirements which would shorten the DNA duplexes needed to clone palindromic sequences. An Alu expression vector with inclusion of a GPF hairpin with its own terminator between two runs of A and including a vector terminator (AluAA-GFP2T) mediates RNAi (Figure 1b). The transcript from this vector should be functionally equivalent to the AluA-GFP vectors with the possible exception that any readthrough of the hairpin terminator will be quickly terminated. A GFP hairpin lacking its own terminator (AluAA-GFP using the GFPnoterm oligos (Table 1)) is able to knock down EGFP in a transient assay (Figure 1b) and in cells selected for episomal AluAA-GFP contructs (Figure 4) To demonstrate the dispensability of the 5' CCCC transcription initiation sequence for H1, we made a GFP targeting hairpin in which those were removed. We called this v1.1. The AluAA-GFPv1.1 maintains activity in our stable knockdown assay (Figure 4).

Other optimizations that work for standard "naked" hairpins were also incorporated into GFP targeting hairpins. RISC shows a preference to incorporate the 5' end of the less stable end of a given siRNA duplex [35;36]. Thus, a less stable loop proximal end in expressed hairpins would more often incorporate the antisense strand. One common optimization of RNA pol III hairpins is to include G:U basepairing in the loop proximal end such that the weaker basepairing destabilizes this end [37]. Another optimization is to include loop sequences from functional microRNAs [38-40]. To design more effective GFP targeting hairpins, we incorporated both of these optimizations into our episomal AluAA expression vector. We used the miR-23 loop sequence [40] in the GFP hairpin using a vector terminator only (AluAA-GFP v2.1GU) and in GFP hairpins including a terminator immediately 3' of the palindromic seqeunces (AluAA-GFP v2.2GU). We found that instead of enhancing activity, these changes abrogated EGFP

knockdown, no EGFP suppression was observed in the first FACS analysis in contrast to AluA-GFP, AluAA-GFP, and AluAA-GFP2T (Figure 4). GFP hairpins with either of these changes showed an intermediate level of EGFP suppression demonstrated that these two changes independently abrogate Alu-hairpin silencing (Figure 4).

Discussion

We have demonstrated stable suppression of an EGFP transgene with BC1(ID)-linked hairpins. The level of knockdown is comparable to H1-expressed "naked" hairpins over the 6 month period. The combination of efficient knockdown combined with less toxicity suggest BC1(ID) would be an excellent carrier for human cell RNAi. While several shRNA vectors that use pol III promoters and/or incorporate endogenous precursors to RNAi have demonstrated robust RNAi, this report is the only demonstration of a stable knockdown vector in which the expression system has been shown to be largely nontoxic in a quantitative assay.

The BC1(ID) long-term RNAi result contrasts with the unexpected observation that target gene suppression by an Alu-hairpin is effectively shut down within 2 months and completely within 4. The only difference between these two vectors is the SINE sequence itself, both are within the same plasmid context and both utilize the 7SL UAS. Several features of Alu might contribute to its silencing. Alu elements show a high C+G content, 63%, in contrast to BC1(ID), 41%. Moreover, our AluYa5 contains 24 CpG dinucleotides and BC1(ID) contains no CpGs. Alu may be a target of methylation leading to silencing as is seen for many transgenes and retroviral promoters [41;42]. Alu, as a human repeat, may also be a target of a specific repeat element silencing pathway which is known to operate in the mammalian germline [43;44]. This vector system is the first demonstration of *de novo* silencing of Alu expression in human cells. Our

creation of an Alu with a specific phenotype (EGFP silencing) could be a useful tool in characterizing the factors that influence Alu expression and/or silencing. Alternatively, there could be some experimental or therapeutic applications of Alu-linked hairpins in which the silencing feature could be useful. Some model or therapeutic systems may require reversal of the RNAi phenotype after an initial repression of the target. One niche use may be in cellular systems in which the silencing mechanism is defective allowing targeted therapies. For examples, many cancer cells show hypomethylation as specifically measured in Alu and LINE-1 repeats [45].

One possibility for the abrogation of hairpin toxicity via SeshRNA is the ability of BC1(ID) and Alu to act as neutral carriers of hairpins. Less expression of hairpins has been associated with less cytotoxic effects [16]. However, that report also demonstrated that the weaker H1 promoter was less effective at mediating RNAi than the stronger U6. Our current and previous data demonstrate that both Alu and BC1(ID) mediate equivalent levels of EGFP and luciferase suppression as H1 under conditions in which H1-hairpins are toxic. One way to account for this effect is to postulate that overexpression of naked hairpins forces the saturation of the RNAi machinery whereas SeshRNA makes hairpins available to the extent that the RNAi machinery can process it. Thus, only a portion of the SeshRNA pool enters the RNAi pathway, and this is essentially a phenocopy of less expression however it apparently still allows maximum efficiency.

We had previously posited that Alu (and now BC1) may be less toxic than other expression systems because it bypasses the normal biogenesis pathway. Exportin-5 is required for miR and "naked" hairpin biogenesis particularly in the export of pri-miRs from the nucleus [19;20]. Vector shRNA expression interferes with endogenous miR silencing, and overexpression of

exportin-5 relieves this titration phenotype although relief of toxicity has not been directly demonstrated [18-20]. Its also likely that Alu or BC1(ID) bypass other siRNA biogenesis factors used for "naked" shRNAs. Observations from this report support the hypothesis that there are mechanistic differences. One, the standard modifications to hairpins to increase incorporation by RISC of the antisense RNA actually abolish activity for Alu-linked hairpins. This suggests that Argonaut2 may not be the active slicer for SeshRNAi. Recently it's been reported that Piwi related proteins perform the ribonuclease function for repeat DNA in Drosophila [46-49]. Alu hairpins may utilize a mammalian repeat associated small interfering RNA (rasiRNA) pathway as well [43;44]. It will be interesting to test which biogenesis pathway factors are required for the conversion of SeshRNA into siRNAs.

The less cytotoxic effects of BC1(ID)-linked hairpins may allow for more reliable and robust analysis in both transient and stable knockdown experiments relative to expression techniques known to cause cells to die. This could be especially important for whole animal experiments; however, the generation of stable SeshRNA knockdown mice is likely to be complicated because neither our Alu nor BC1(ID) stable constructs mediate EGFP knockdown in mouse 3T3 or in CHO cells (data not shown). Whether this lack of suppression is inherent to rodent cells or particular to these individual cell lines will have to be determined. But currently in regard to human cell line tissue culture, it appears the SeshRNA is an excellent technique for DNA directed RNAi that combines efficacy with demonstrated less cytotoxicity.

Acknowledgments

Reference List

1. Tomari,Y. and P.D.Zamore. 2005. Perspective: machines for RNAi. Genes Dev 19:517-529.

2. Hannon,G.J. 2002. RNA interference. Nature 418:244-251.

3. Snove,O., Jr. and J.J.Rossi. 2006. Expressing short hairpin RNAs in vivo. Nat Methods 3:689-695.

4. Brummelkamp,T.R., R.Bernards and R.Agami. 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science 296:550-553.

5. Koper-Emde,D., L.Herrmann, B.Sandrock and B.J.Benecke. 2004. RNA interference by small hairpin RNAs synthesised under control of the human 7S K RNA promoter. Biol Chem 385:791-794.

6. Paddison,P.J., A.A.Caudy, E.Bernstein, G.J.Hannon and D.S.Conklin. 2002. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 16:948-958.

7. Paul,C.P., P.D.Good, I.Winer and D.R.Engelke. 2002. Effective expression of small interfering RNA in human cells. Nat Biotechnol 20:505-508.

8. Sui,G., C.Soohoo, e.B.Affar, F.Gay, Y.Shi, W.C.Forrester and Y.Shi. 2002. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci U S A 99:5515-5520.

9. Yu,J.Y., S.L.DeRuiter and D.L.Turner. 2002. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. Proc Natl Acad Sci U S A 99:6047-6052.

10. Bernstein,E., A.A.Caudy, S.M.Hammond and G.J.Hannon. 2001. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409:363-366.

11. Abbas-Terki,T., W.Blanco-Bose, N.Deglon, W.Pralong and P.Aebischer. 2002. Lentiviral-mediated RNA interference. Hum Gene Ther 13:2197-2201.

12. Pebernard,S. and R.D.Iggo. 2004. Determinants of interferon-stimulated gene induction by RNAi vectors. Differentiation 72:103-111.

13. Cao,W., R.Hunter, D.Strnatka, C.A.McQueen and R.P.Erickson. 2005. DNA constructs designed to produce short hairpin, interfering RNAs in transgenic mice sometimes show early lethality and an interferon response. J Appl Genet 46:217-225.

14. Bridge,A.J., S.Pebernard, A.Ducraux, A.L.Nicoulaz and R.Iggo. 2003. Induction of an interferon response by RNAi vectors in mammalian cells. Nat Genet 34:263-264.

15. Fish,R.J. and E.K.Kruithof. 2004. Short-term cytotoxic effects and long-term instability of RNAi delivered using lentiviral vectors. BMC Mol Biol 5:9.

16. An,D.S., F.X.Qin, V.C.Auyeung, S.H.Mao, S.K.Kung, D.Baltimore and I.S.Chen. 2006. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. Mol Ther 14:494-504.

17. Gasior,S.L., M.Palmisano and P.L.Deininger. 2006. Alu-linked hairpins efficiently mediate RNA interference with less toxicity than do H1-expressed short hairpin RNAs. Anal Biochem 349:41-48.

18. Grimm,D., K.L.Streetz, C.L.Jopling, T.A.Storm, K.Pandey, C.R.Davis, P.Marion, F.Salazar and M.A.Kay. 2006. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441:537-541.

19. Yi,R., B.P.Doehle, Y.Qin, I.G.Macara and B.R.Cullen. 2005. Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs. RNA 11:220-226.

20. Yi,R., Y.Qin, I.G.Macara and B.R.Cullen. 2003. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17:3011-3016.

21. Pillai,R.S., S.N.Bhattacharyya and W.Filipowicz. 2007. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 17:118-126.

22. Cullen,B.R. 2004. Transcription and processing of human microRNA precursors. Mol Cell 16:861-865.

23. Zhou,H., X.G.Xia and Z.Xu. 2005. An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. Nucleic Acids Res 33:e62.

24. Stegmeier,F., G.Hu, R.J.Rickles, G.J.Hannon and S.J.Elledge. 2005. A lentiviral microRNA-based system for single-copy polymerase II-regulated RNA interference in mammalian cells. Proc Natl Acad Sci U S A 102:13212-13217.

25. Chung,K.H., C.C.Hart, S.Al Bassam, A.Avery, J.Taylor, P.D.Patel, A.B.Vojtek and D.L.Turner. 2006. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. Nucleic Acids Res 34:e53.

26. Kawasaki,H. and K.Taira. 2003. Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. Nucleic Acids Res 31:700-707.

27. Scherer,L.J., R.Frank and J.J.Rossi. 2007. Optimization and characterization of tRNA-shRNA expression constructs. Nucleic Acids Res 35:2620-2628.

28. Kobayashi,S., T.Higuchi and K.Anzai. 2005. Application of the BC1 RNA gene promoter for short hairpin RNA expression in cultured neuronal cells. Biochem Biophys Res Commun 334:1305-1309.

29. Paul,C.P. 2005. Subcellular distribution of small interfering RNA: directed delivery through RNA polymerase III expression cassettes and localization by in situ hybridization. Methods Enzymol 392:125-145.

30. Paul,C.P., P.D.Good, S.X.Li, A.Kleihauer, J.J.Rossi and D.R.Engelke. 2003. Localized expression of small RNA inhibitors in human cells. Mol Ther 7:237-247.

31. Borchert,G.M., W.Lanier and B.L.Davidson. 2006. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol 13:1097-1101.

32. Roy,A.M., N.C.West, A.Rao, P.Adhikari, C.Aleman, A.P.Barnes and P.L.Deininger. 2000. Upstream flanking sequences and transcription of SINEs. J Mol Biol 302:17-25.

33. Kim,J., J.A.Martignetti, M.R.Shen, J.Brosius and P.Deininger. 1994. Rodent BC1 RNA gene as a master gene for ID element amplification. Proc Natl Acad Sci U S A 91:3607-3611.

34. Rozhdestvensky,T.S., A.M.Kopylov, J.Brosius and A.Huttenhofer. 2001. Neuronal BC1 RNA structure: evolutionary conversion of a tRNA(Ala) domain into an extended stem-loop structure. RNA 7:722-730.

35. Khvorova,A., A.Reynolds and S.D.Jayasena. 2003. Functional siRNAs and miRNAs exhibit strand bias. Cell 115:209-216.

36. Schwarz,D.S., G.Hutvagner, T.Du, Z.Xu, N.Aronin and P.D.Zamore. 2003. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115:199-208.

37. Medema,R.H. 2004. Optimizing RNA interference for application in mammalian cells. Biochem J 380:593-603.

38. Miyagishi,M., H.Sumimoto, H.Miyoshi, Y.Kawakami and K.Taira. 2004. Optimization of an siRNA-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. J Gene Med 6:715-723.

39. Boden,D., O.Pusch, R.Silbermann, F.Lee, L.Tucker and B.Ramratnam. 2004. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. Nucleic Acids Res 32:1154-1158.

40. Xia,H., Q.Mao, S.L.Eliason, S.Q.Harper, I.H.Martins, H.T.Orr, H.L.Paulson, L.Yang, R.M.Kotin and B.L.Davidson. 2004. RNAi suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. Nat Med 10:816-820.

41. Lorincz,M.C., D.Schubeler, S.C.Goeke, M.Walters, M.Groudine and D.I.Martin. 2000. Dynamic analysis of proviral induction and De Novo methylation: implications for a histone deacetylase-independent, methylation density-dependent mechanism of transcriptional repression. Mol Cell Biol 20:842-850.

42. Swindle,C.S., H.G.Kim and C.A.Klug. 2004. Mutation of CpGs in the murine stem cell virus retroviral vector long terminal repeat represses silencing in embryonic stem cells. J Biol Chem 279:34-41.

43. Aravin,A.A., R.Sachidanandam, A.Girard, K.Fejes-Toth and G.J.Hannon. 2007. Developmentally regulated piRNA clusters implicate MILI in transposon control. Science 316:744-747.

44. Carmell,M.A., A.Girard, H.J.van de Kant, D.Bourc'his, T.H.Bestor, D.G.de Rooij and G.J.Hannon. 2007. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. Dev Cell 12:503-514.

45. Schulz,W.A., C.Steinhoff and A.R.Florl. 2006. Methylation of endogenous human retroelements in health and disease. Curr Top Microbiol Immunol 310:211-250.

46. Girard,A., R.Sachidanandam, G.J.Hannon and M.A.Carmell. 2006. A germline-specific class of small RNAs binds mammalian Piwi proteins. Nature 442:199-202.

47. Vagin,V.V., A.Sigova, C.Li, H.Seitz, V.Gvozdev and P.D.Zamore. 2006. A distinct small RNA pathway silences selfish genetic elements in the germline. Science 313:320-324.

48. Saito,K., K.M.Nishida, T.Mori, Y.Kawamura, K.Miyoshi, T.Nagami, H.Siomi and M.C.Siomi. 2006. Specific association of Piwi with rasiRNAs derived from retrotransposon and heterochromatic regions in the Drosophila genome. Genes Dev 20:2214-2222.

49. Kalmykova,A.I., M.S.Klenov and V.A.Gvozdev. 2005. Argonaute protein PIWI controls mobilization of retrotransposons in the Drosophila male germline. Nucleic Acids Res 33:2052-2059.

Table I: Oligonucleotides

Name	Seq
GFP_v1.1_s	GATCTGAACGGCATCAAGGTGAACTTCAAGAGAGTTCACCTTGATGCCGTTCTTTTTA
GFP_v1.1_as	AGCTTAAAAAGAACGGCATCAAGGTGAACTCTCTTGAAGTTCACCTTGATGCCGTTCA
GFP_loopGU_s	GATCTAAGAACGGCATCAGGGTGGACCTTCCTGTCAGTTCACCTTGATGCCGTTCTTCA
GFP_loopGU_as	AGCTTGAAGAACGGCATCAAGGTGAACTGACAGGAAGGTCCACCCTGATGCCGTTCTTA
GFP_loopGU2T_s	GATCTGAACGGCATCAGGGTGGACCTTCCTGTCAGTTCACCTTGATGCCGTTCTTTTTGGAAA
GFP_loopGU2T_as	AGCTTTTCCAAAAAGAACGGCATCAAGGTGAACTGACAGGAAGGTCCACCCTGATGCCGTTCA
GFP_GU1_s	GATCCGAACGGCATCAAGGTGAGCTTCAAGAGAGTTCACCTTGATGCCGTTCA
GFP_ GU1_as	AGCTTGAACGGCATCAAGGTGAACTCTCTTGAAGCTCACCTTGATGCCGTTCG
GFP_GU2_s	GATCCGAACGGCATCAAGGTGGATTTCAAGAGAGTTCACCTTGATGCCGTTCA
GFP_ GU2_as	AGCTTGAACGGCATCAAGGTGAACTCTCTTGAAATCCACCTTGATGCCGTTCG
GFP_miRLoop_s	GATCCGAACGGCATCAAGGTGAACCTTCCTGTCAGTTCACCTTGATGCCGTTCA
GFP_ miRLoop _as	AGCTTGAACGGCATCAAGGTGAACTGACAGGAAGGTTCACCTTGATGCCGTTCG
2ndA_s	AGCTTAAAAAAAAAAAAAAAAAAAAATTTTTT
2ndA _as	CTAGAAAAAATTTTTTTTTTTTTTTTTTTTTTA

Figure 1: BC1 is relatively nontoxic in HeLa cells and mediates RNAi of EGFP. a) The indicated shRNA expression constructs with associated hairpins or terminator were transfected into Us9-EGFP-expressing HeLa cells and analyzed by FACS for EGFP fluorescence intensity. pSuper_AluAA includes a terminator 3' of the 20A run that is 3' of the cloning site. AluAA-GFP includes a cloned hairpin sequence without its own terminator (oligoes for cloning were GFPnoterm, Table 1) whereas AluAA-GFP2T includes the standard terminator-containing hairpin. The starting population was approximately 70% EGFP+ after a second FACS selection of a previously used cell line (Greenish2, M&M). The figure demonstrates the quantitation for each sample as a percentage of viable cells that are EGFP+. b) 2.4 µg of the various pSuper vectors (either empty vector, vector with terminator (-term), or EGFP (GFP) hairpins) or pSuper backbone with the 7SL-BC1 expression construct were cotransfected into HeLa cells with a G418^r expression vector (pIRES2-EGFP) and assayed for colony formation after 2 weeks under Geneticin selection. The relative number of colonies compared to the pSuper-term vector is shown for each construct. n=3. Error bars represent the standard error.

Figure 2: Stable RNAi of EGFP by BC1(ID). A) HeLa cells were transfected with an EGFP expression cassette and selected for G418r. After two rounds of FACS selection for EGFP+ cells (greenish 2), the majority of cells become EGFP- after months of passaging. Clonal selection yielded cells that uniformly and stably express EGFP (one clone, H, was used for further analysis). b) The indicated shRNA episomal expression constructs were transfected into Us9-EGFP-expressing HeLa cells (greenish2 clone H) and analyzed by FACS for EGFP fluorescence intensity. Sample histograms of EGFP expressing cells 3 weeks after transfection and hygromycin selection are presented. Note that the H1-GFP profile used fewer cells than normal
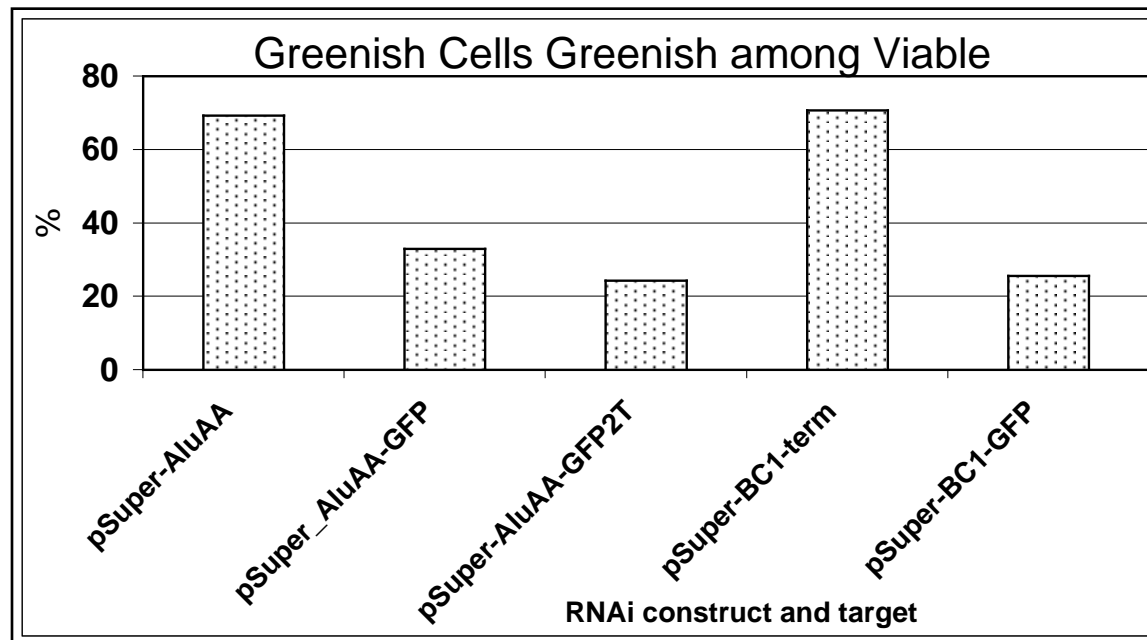
due to a small fraction of viable cells. Outgrowth corrected this problem for future analyses. c) Timecourse of EGFP expression for each sample showing the percentage of viable cells that are EGFP positive. d) The same as b) but 6 months after transfection. e) A second timecourse of EGFP expression for each sample from a second set of transfections showing the percentage of viable cells that are EGFP positive.

Figure 3: RNAi of EGFP by Alu under Selection. a) The indicated shRNA expression constructs were transfected into Us9-EGFP-expressing HeLa cells (greenish2 clone H) which underwent hygromycin selection. Cells were analyzed by FACS for EGFP fluorescence intensity. Sample histograms of EGFP expressing cells 3 weeks after transfection and hygromycin selection with the noted shRNA expression vectors. b) The same as a) but at 6 months after transfection. c and d) Quantitation of timecourses of three separate experiments for each sample as a percentage of viable cells that are EGFP positive.

Figure 4: Abrogation of RNAi of EGFP by modification of Alu-linked hairpins. The indicated episomal Alu shRNA expression constructs were transfected into Us9-EGFP-expressing HeLa cells (greenish2 clone H) which underwent hygromycin selection, and analyzed by FACS for EGFP fluorescence intensity. Quantitation of samples are presented as a percentage of viable cells that are EGFP positive. AluA-GFP and AluAA-GFP2T: standard hairpin configuration. AluAA-GFP: cloned with GFP_noterm oligoes and does not include a terminator in the cloning oligoes. AluAA-GFPv1.1: eliminates CCCC at 5' end of the standard GFP hairpin. AluAA-GFP-loopGU: modified GFPnoterm with miR-23 loop, inclusion of 2 GU mismatches, and elimination of 5' CCCC. AluAA-GFP-loopGU2T: as previous but with terminator included in cloning oligoes. AluAA-GFP GU1/GU2: modified GFP-loopGU with inclusion of one or two GU mismatches but the standard H1 loop sequences. AluAA-GFP miRLoop: modified GFP-loopGU with no GU mismatches.

Figure 1: BC1 is relatively nontoxic in HeLa cells and mediates RNAi of EGFP.
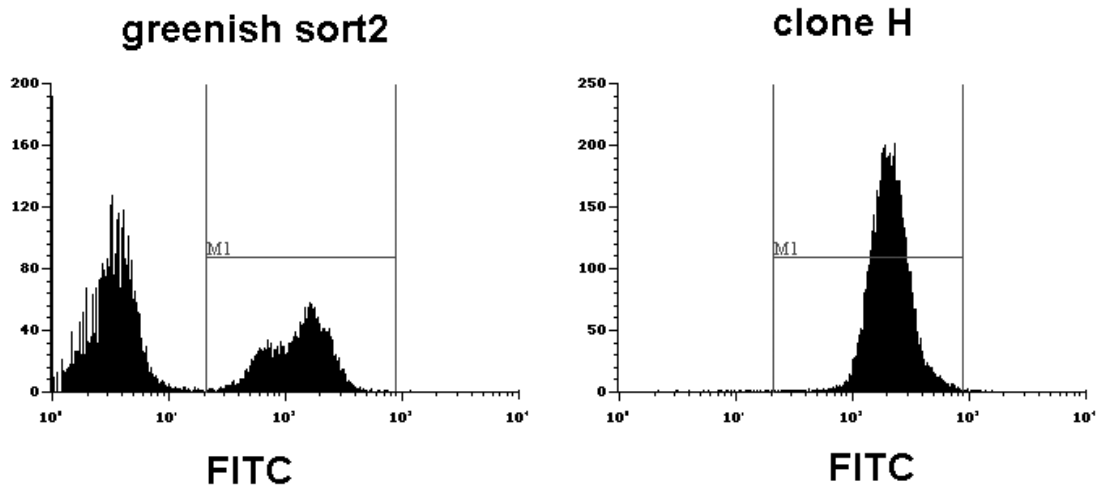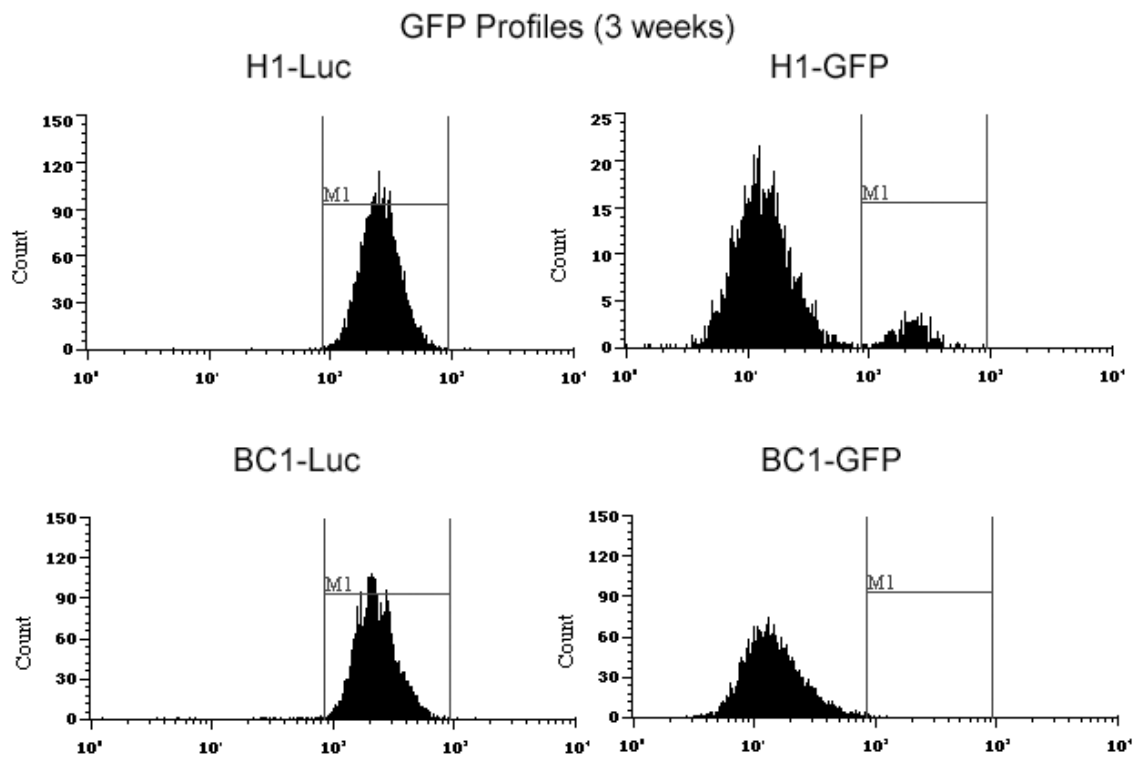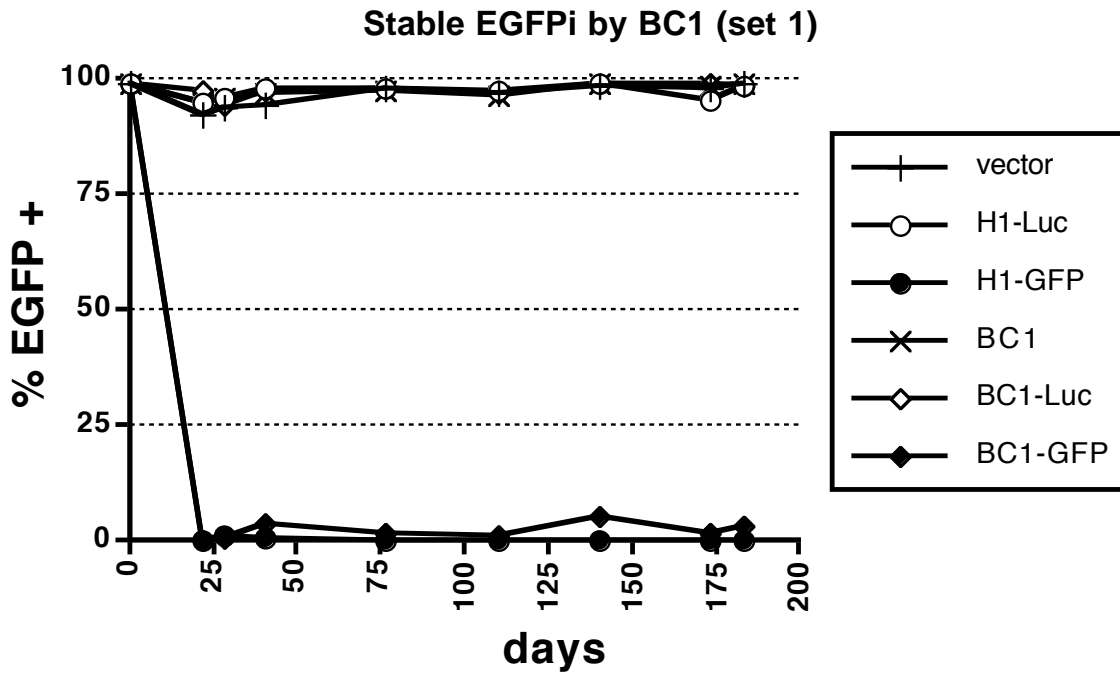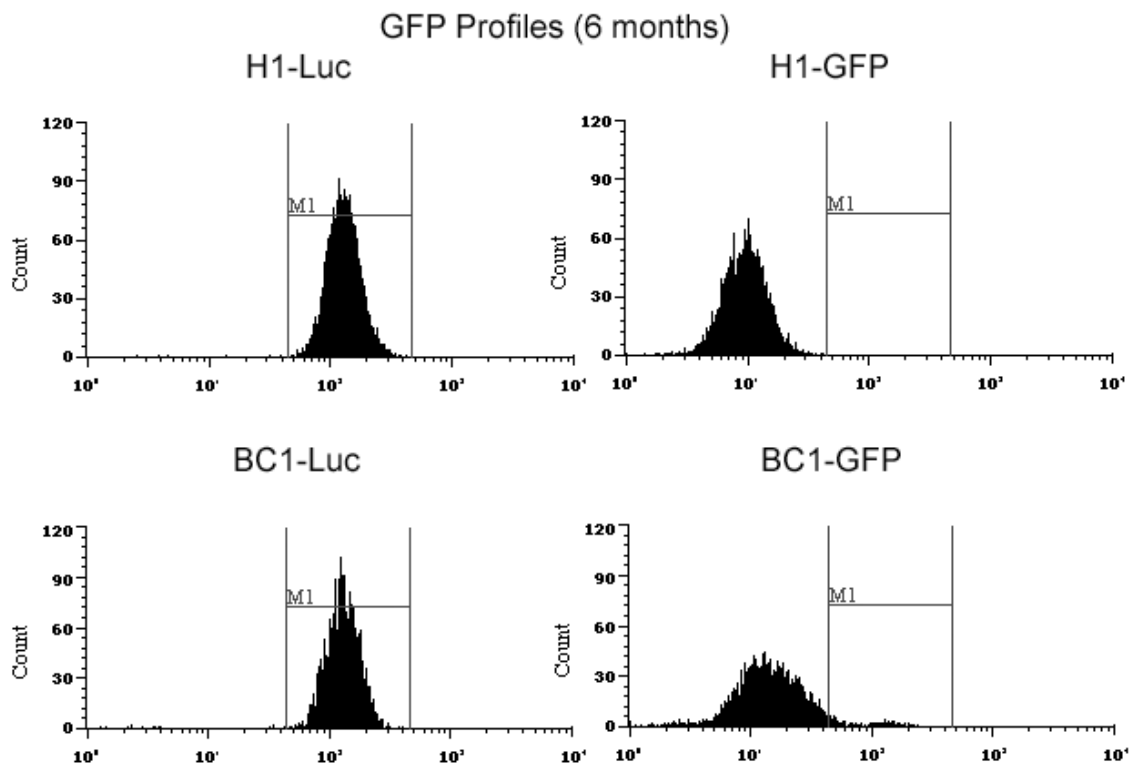
a

b

Figure 2: Stable RNAi of EGFP by BC1(ID).

a

b

c

Stable EGFPi by BC1 (set 1)
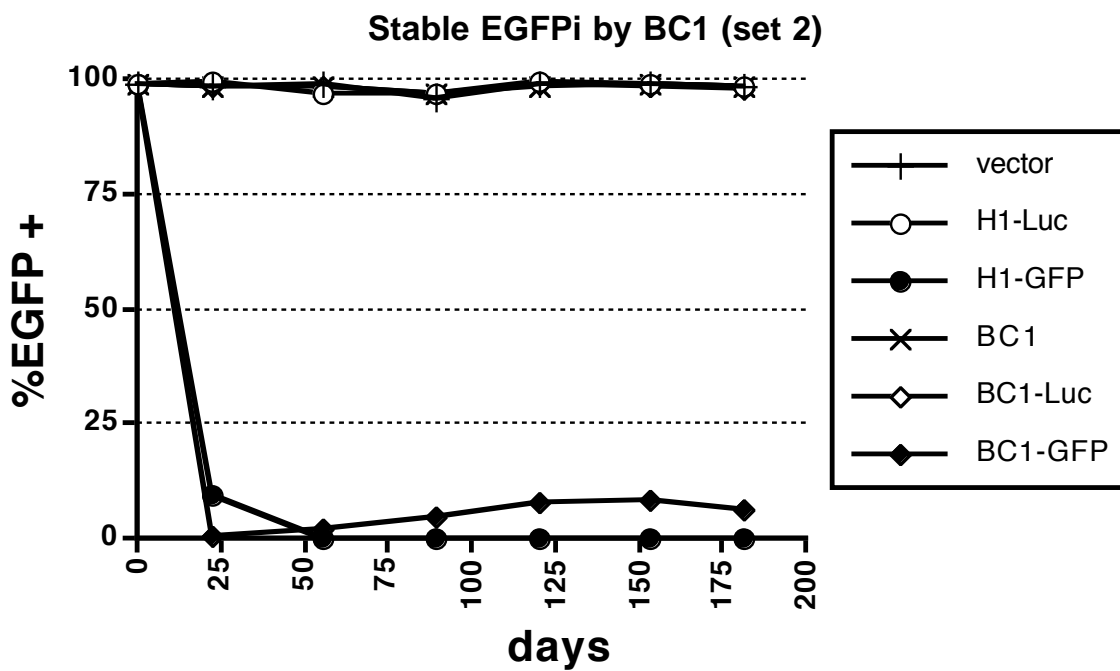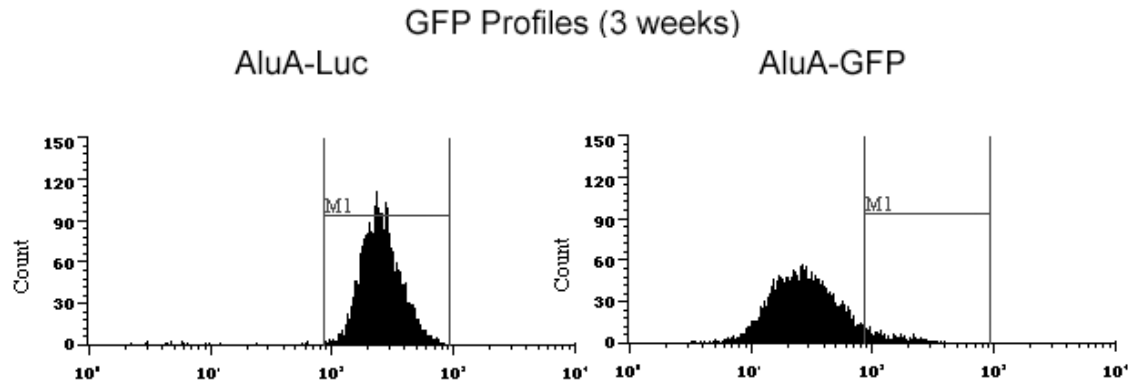

d

GFP Profiles (6 months)

e

Stable EGFPi by BC1 (set 2)

Figure 3: RNAi of EGFP by Alu under Selection.

a

b

c

EGFPi by Alu (set 1)

Legend:
- □ AluA-Luc
- ■ AluA-GFP
- ✕ AluA
- ⊞ AluA-term

Y-axis: %EGFP +
X-axis: days

d

EGFPi by Alu (sets 2+3)

Legend:
- △ AluA-term (2)
- ▲ AluA-GFP (2)
- ⊞ AluA-term (3)
- ⊞ AluA-GFP (3)

Y-axis: %EGFP+
X-axis: days
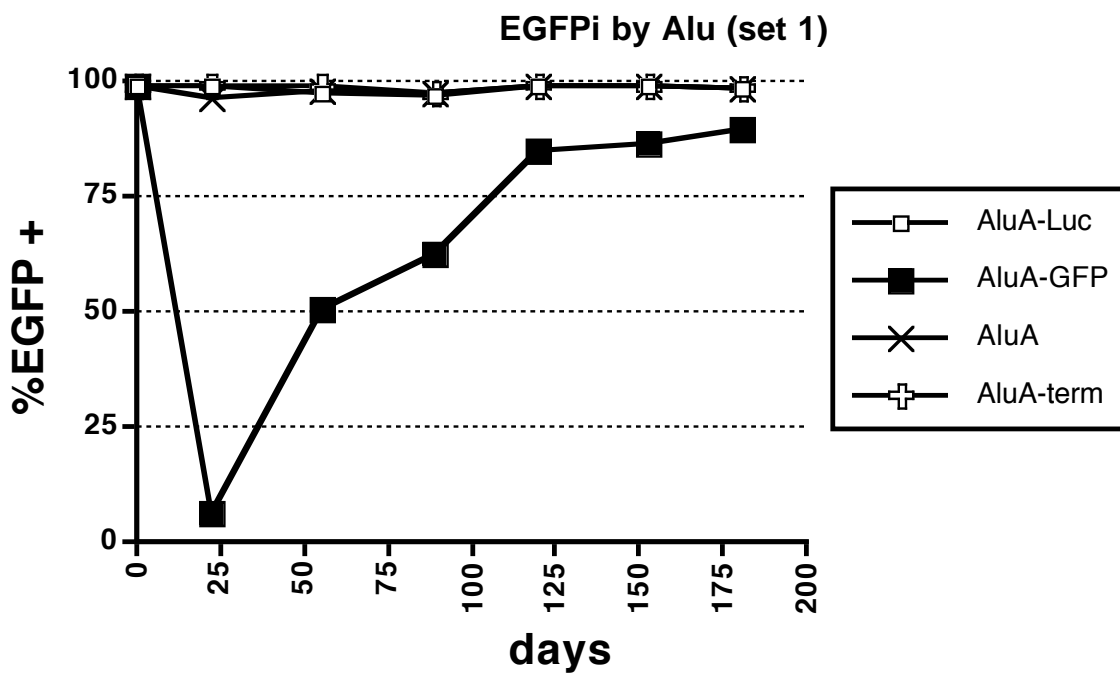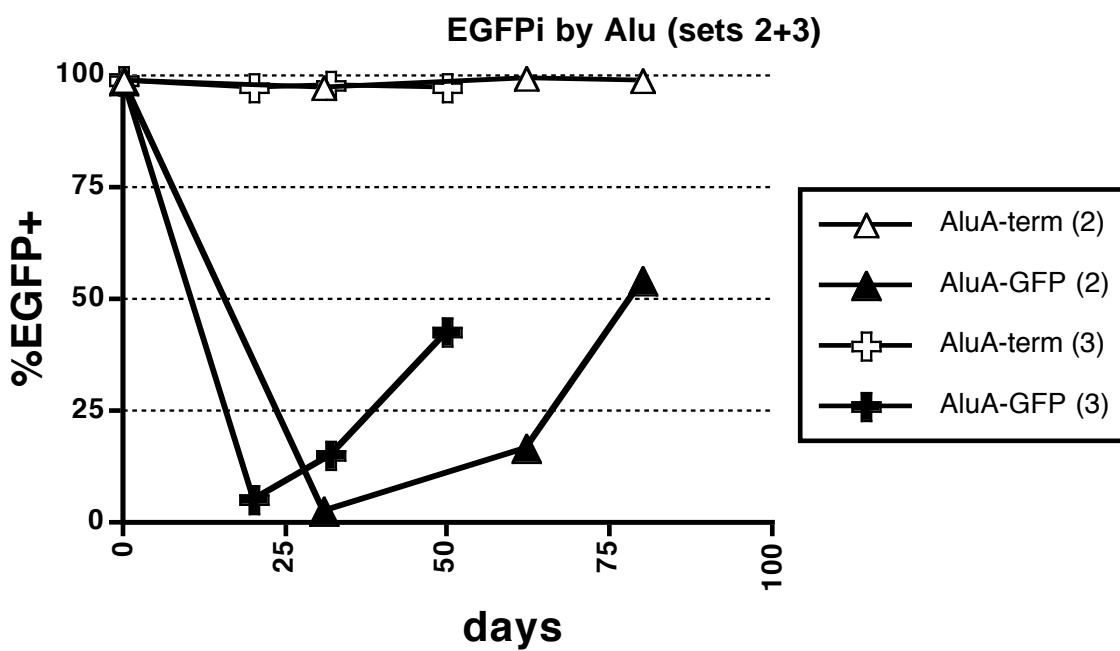
Figure 4: Abrogation of RNAi of EGFP by modification of Alu-linked hairpins.

EGFPi after selection

% EGFP+

RNAi construct and target

AluA-term, AluA-GFP, AluAA, AluAA-GFP, AluAA-GFP2T, AluAA-GFPv1.1, AluAA-loopGU, AluAA-GFP-loopGU2T, AluAA-GFP GU1, AluAA-GFP GU2, AluAA-GFP miRLoop