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14. ABSTRACT
We have successfully prepared sense expression libraries, characterized the size of their inserts which are fused to a green fluorescent protein tag, and packaged these libraries into lentivirus. Four different human MDS bone marrow isolates have been transfected with lentivirus at multiplicity of infection of 0.1. A collection of 252 puromycin resistant clones were isolated, genomic DNA was prepared, and clones were screened for ability to undergo myeloid differentiation in response to GM-CSF. Only ~30% of the puromycin resistant clones (78) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 78 clones. DNA sequencing has identified the cDNA inserts. These inserts were expressed in naïve MDS cell pools. Eight of these cDNAs were validated; they induced myeloid colonies in vitro and engrafted in the marrow of SG3, but not NSG mice.

15. SUBJECT TERMS
Myelodysplastic syndrome, lentivirus, cDNA libraries, complementation

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Introduction

Rationale: The focus of our proposal is the identification of normal human genes, that when overexpressed by transduction of a lentiviral vector, complement the underlying genetic defect in MDS cells. Our alternate approach was to downregulate gene expression using shRNA libraries, and identify genes whose overexpression promote the MDS phenotype. Our ultimate endpoint is the detection of terminal differentiation and colony formation resulting in normal myeloid cells.

Hypothesis: Expression of a normal human cDNA library in MDS cells will correct the aberrant phenotype and permit normal proliferation and myeloid differentiation in selected clones.

Body

In this study we test the assumption that over-expression of a wild type gene, or suppression of an abnormally expressed gene by shRNA, in a transfected MDS clone will interrupt the pathogenic signaling that gives rise to the MDS phenotype. Therefore, the transfected gene will permit normal differentiation of the MDS cell into a normal myeloid lineage. The first objective (months 1 – 6 in SOW) was to prepare and characterize the lentivirus libraries.

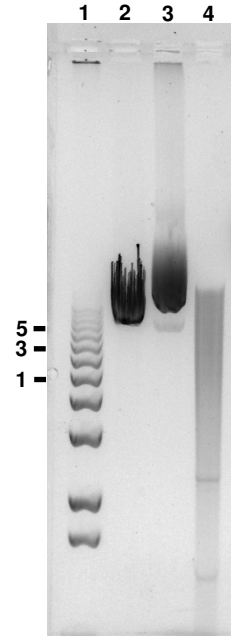


Fig. 1. Construction of human liver cDNA expression library. Library contains ~7 x 10^7 individual clones, and the average insert size is >2 kb. Lane 1: marker, size indicated in kb; Lane 2: linearized plasmid; Lane 3: plasmid and ligated insert; Lane 4: human liver cDNA insert prior to ligation.

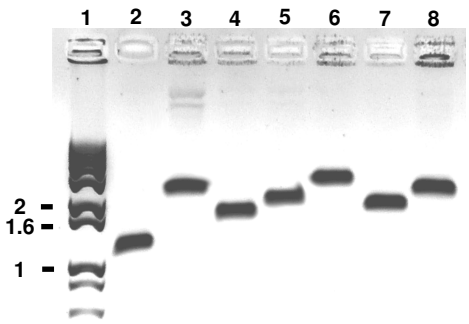
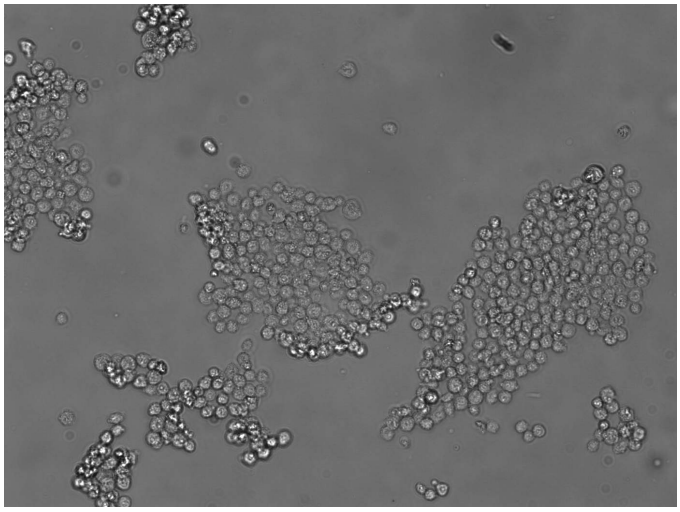


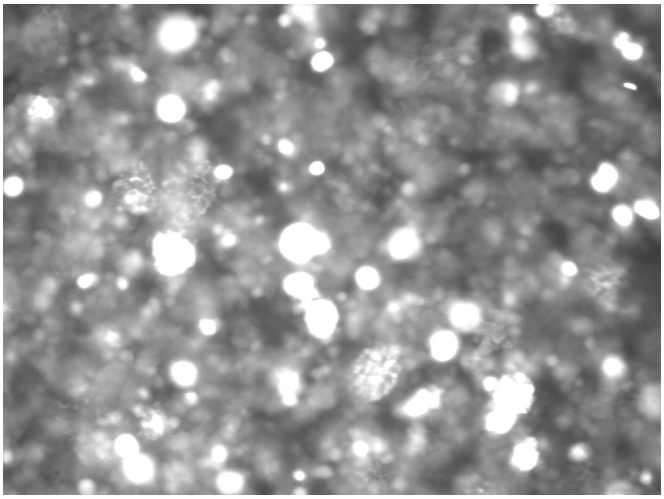
Fig. 2. Characterization of lentivirus cDNA expression library. Following packaging and titering of the lentivirus preparation, 293T cells were infected with library at multiplicity of infection of 0.1 and selected with puromycin x 1 wk. Individual surviving clones were expanded over 10 days and screened by PCR using LTR-specific primers. Lane 1: marker, size indicated in kb; Lanes 2 - 8: PCR product from seven individual clones.

Fig. 3. Transfection of human MDS cells with lentivirus cDNA expression library. MDS bone marrow cells from 5q- (pictured at right), mono 7 / 7q-, trisomy 8, and del 20q were transfected at an moi of 0.1 with the lentivirus library described above. After 14 days growth in soft agar surviving colonies were harvested, dissociated, replated as replicates, and aliquots were cryopreserved. Cells transfected with empty lentivirus particles did not generate proliferating colonies. 200x



Our next goal was to transfect human MDS cells with lentivirus, select for survivors with puromycin, and test for expression of cDNAs contained in the library. Surviving colonies were dissociated, plated as replicas, and aliquots were cryopreserved for future murine studies. Replated colonies were tested for their proliferative and differentiative response to GM-CSF (months 3 – 9 in SOW).

Fig. 4. Detection of lentivirus-encoded GFP in transfected human MDS cells. cDNA inserts encode GFP fusion proteins. Fluorescence microscopy demonstrated strong expression of GFP in puromycin-resistant surviving colonies. 200x



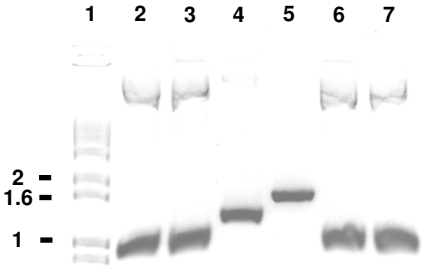


Fig. 5. Expression of lentivirus-encoded cDNA-GFP fusion proteins in transfected human MDS cells. Six GFP-positive colonies were isolated and screened by PCR using LTR-specific primers to demonstrate that fluorescent MDS cells also retained expression of the encoded library-derived cDNA.

A total of 252 puromycin-resistant 7q- clones were isolated, genomic DNA was prepared, and clones were screened for ability to undergo myeloid differentiation in response to GM-CSF in vitro. Only ~30% of the puromycin resistant clones (78) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 78 clones. These inserts were PCR amplified from isolated colonies, packaged into lentivirus and used to infect naïve MDS cell pools. Eight of these cDNAs were validated; that is, they induced myeloid colonies in transfected MDS cells vitro. Sequencing identified the cDNA inserts (Table 1).

Table 1	
Symbol	Description
HRAS	Homo sapiens v-Ha-ras Harvey rat sarcoma viral oncogene homolog (HRAS), transcript 1
CDC25C	Homo sapiens cell division cycle 25 homolog C (CDC25C), transcript variant 1
MYC	Homo sapiens v-myc myeloctomatosis viral oncogene homolog (avian) (MYC)
MAP3K7	Homo sapiens mitogen-activated protein kinase kinase kinase 7 (MAP3K7)
MAP3K8	Homo sapiens mitogen-activated protein kinase kinase kinase 8 (MAP3K8)
SF3B1	Homo sapiens splicing factor 3b, subunit 1, 155kDa (SF3B1), transcript variant 1
SIK1	Homo sapiens salt-inducible kinase 1 (SIK1)
TET2	Homo sapiens tet oncogene family member 2 (TET2), transcript variant 2

Sequence data for these clones is attached in appendix 1. The SF3B1 and TET2 clones both encoded only 5' regions of the proteins (64% and 41%, respectively). The yellow highlighting indicates identical regions of the clones we isolated and the corresponding regions of the full length NCBI cDNA sequence. The fact that these two clones contained only partial sequence may be due to the fact that both of the cDNAs are quite large (3912 bp and 6006 bp, respectively) and the reverse transcription reactions during library synthesis may not have gone to completion. Alternatively, there may be steric restriction due to packaging constraint within the viral capsid. The other six clones contained complete cDNA for encoded proteins.

The next objective was to characterize surface markers and phenotype the successful transfectants (months 6 – 12 in SOW). Normal bone marrow cells have a low percentage of blasts (~2-3%), represented by gated population (Fig. 7, black circle). This gated population has low expression of c-kit and CD34 in normal marrow. MDS marrow with elevated blasts (lower panels) have high expression of c-kit and CD34. By flow cytometry, the expression of c-kit, CD34, CD33, CD13, HLA-DR was unchanged in our collection of eight lentivirus-transfected MDS isolates when compared to mock-transfected or empty virus transfected cells.

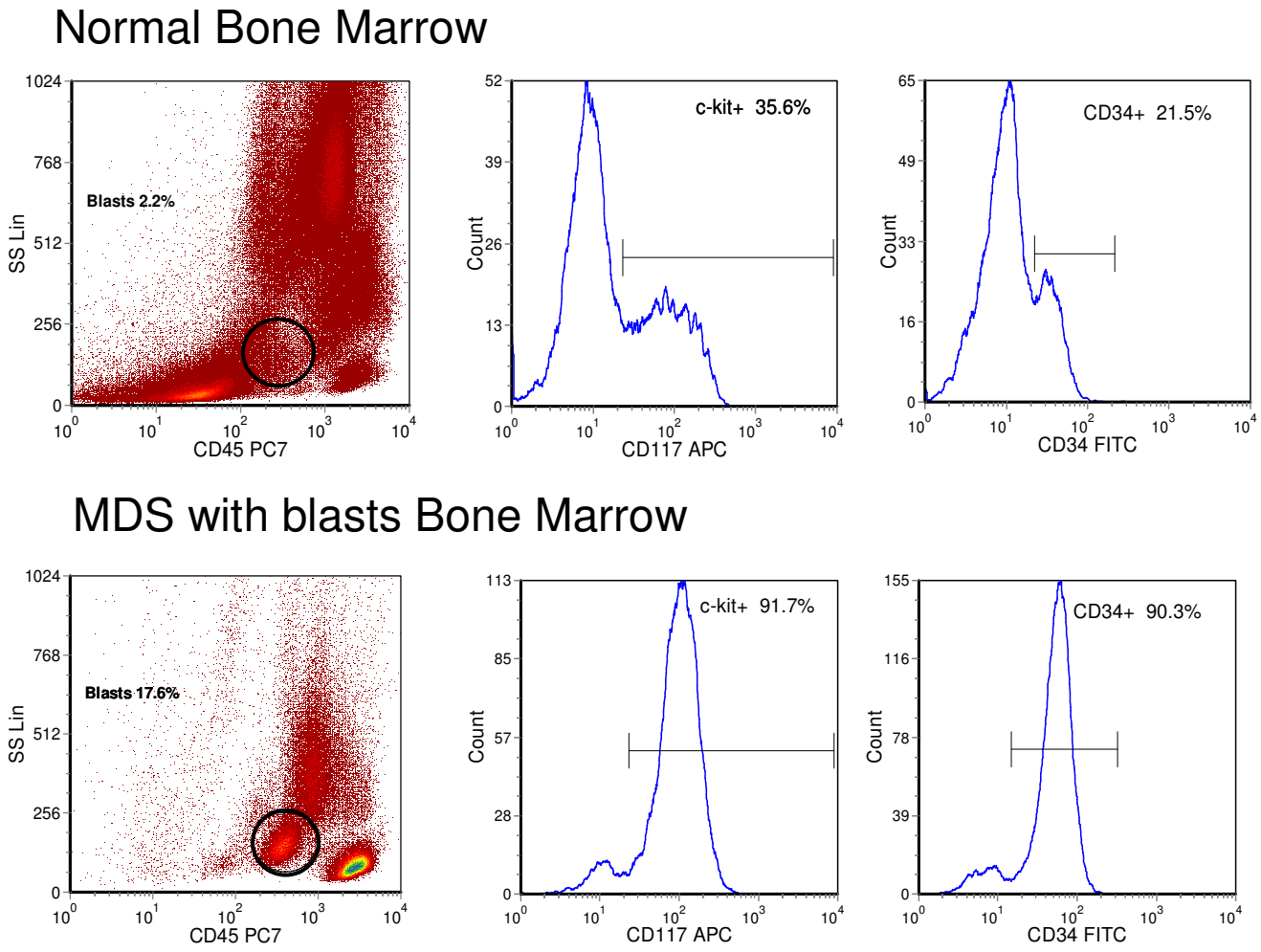


Fig. 7. Characterization of MDS surface markers. Normal human bone marrow (upper panels), and 7q- MDS marrow (lower panels) was stained for CD45 (leukocyte common antigen), CD34 (stem cell marker), and CD117 (c-kit). Cells were also $CD13^+$ $CD33^+$ $HLA\text{-}DR^+$. Patient was characterized as refractory anemia with excess blasts II (RAEB-II) (10-19% blasts), Auer rods negative.

We inoculated NOD.Cg-*Prkdcscid Il2rgtm1Wjl*/SzJ mice (NSG mice) (1-8) with 7q- transfectants expressing the nine candidate genes. Our objective was to test ability of transfectants to engraft NSG mice and generate chimeric bone marrow (months 12 – 18). NSG mice did not engraft well with transfected human normal or MDS marrow. Normal human marrow and MDS marrow was detectable in NSG mice up to 45 days at 1 – 4%, and was not detectable in peripheral blood. Engraftment was defined as presence of >5% human CD45 cells in murine marrow sixty days after inoculation. Therefore, we utilized SG3 mice (Jackson Laboratories), in which NSG mice express three human trans genes: stem cell factor, GM-CSF, and IL-3, all of which support myeloid growth and differentiation (9). Mice were sacrificed at 3 and 6 months following inoculation with transfected MDS cells to evaluate the extent of engraftment. The transfected clones were all able to engraft SG3 mice to varying degrees and persisted in marrow up to 6 months (Table 2). Empty lentivirus-transfected MDS cells (Null) were unable to engraft murine marrow. In no case were human CD45 cells detectable in peripheral blood.

Table 2				
Symbol	Chromosome	% engraftment (human CD45 vs. murine)	Presence of human CD45 in peripheral blood	Maximum duration of marrow engraftment (mo)
Null	-	0	-	0
HRAS	11p	35	-	6
CDC25C	5q	27	-	3
MYC	8q	41	-	3
MAP3K7	6q	14	-	6
MAP3K8	10p	11	-	3
SF3B1	2q	19	-	6
SIK1	21q	13	-	3
TET2	4q	20	-	6

Key Research Accomplishments

1. Creation of cDNA lentivirus libraries
2. Transfection of human MDS bone marrow cells with lentivirus libraries
3. Expression of cDNA-GFP fusion proteins in MDS cells
4. Identification of eight cDNAs that drive myeloid differentiation in MDS cells
5. Characterization of markers associated with MDS blasts

Reportable Outcomes

This data was reported at the 2011 Case Comprehensive Cancer Center Retreat, Corporate College East, July 8, 2011.

Abstract: Complementation of Myelodysplastic Syndrome Clones with Lentivirus Expression Libraries. Daniel Lindner, Translational Hematology & Oncology Research, Taussig Cancer Institute, Cleveland Clinic.
The goal of these studies was to identify genes that when expressed, would permit human myelodysplastic syndrome (MDS) bone marrow cells to undergo normal colony formation in vitro and engraft the marrow of NSG mice. Utilizing sense expression cDNA libraries generated from normal human bone marrow, inserts fused in frame to green fluorescent protein tag were packaged into lentivirus. Four different human MDS bone marrow isolates were transfected with lentivirus at multiplicity of infection of 0.1. A collection of 252 puromycin resistant clones were isolated, genomic DNA was prepared, and clones were screened for ability to

undergo myeloid differentiation in response to GM-CSF. Approximately 30% of the puromycin resistant clones (78) acquired this phenotype. Transfection of MDS cells with antisense libraries did not generate any clones that acquired the desired (differentiative) phenotype. PCR was used to identify presence of the trans gene in the 78 clones. DNA sequencing has identified the cDNA inserts. These inserts were expressed in naïve MDS cell pools. Eight of these cDNAs were validated; they induced myeloid colonies in vitro and engrafted in the marrow of SG3, but not NSG mice.

This data was presented at the 2012 Case Comprehensive Cancer Center NCI P30 Site Visit, Corporate College East, September 7, 2012.

Abstract: Complementation of Myelodysplastic Syndrome Clones Permits Normal Myeloid Colony Formation and NSG Mouse Marrow Engraftment. Daniel Lindner, Translational Hematology & Oncology Research, Taussig Cancer Institute, Cleveland Clinic.

The goal of these studies was to validate eight genes that when expressed in human myelodysplastic syndrome (MDS) bone marrow cells, resulted in normal myeloid CFU formation in vitro and engraftment of murine bone marrow. Utilizing sense expression cDNA libraries generated from normal human bone marrow, inserts fused in frame to green fluorescent protein tag were packaged into lentivirus. MDS bone marrow isolates were transfected with lentivirus at multiplicity of infection of 0.1. A collection of puromycin resistant clones were isolated and screened for ability to undergo myeloid differentiation in response to GM-CSF. Eight cDNAs were expressed in naïve MDS cell pools and their functional properties validated: that is, they induced myeloid colonies in vitro and engrafted in the marrow of SG3, but not NSG mice. These cDNAs encoded HRAS, CDC25C, MYC, MAP3K7, MAP3K8, SF3B1, SIK1, and TET2.

Conclusion

We have identified eight genes that when overexpressed, conferred proliferative and differentiative capabilities to MDS isolates. Some of these were found to be cellular oncogenes such as Ras, Myc, and Tet2. Their identification in this genetic screen give support to the validity of the method. Another candidate, CDC25C is a tyrosine phosphatase that drives cellular entry into mitosis. The mitogen-activated protein kinase kinase kinase 7 (MAP3K7) controls transcriptional regulation, whereas MAP3K8 may function as an oncogene. SIK1 is a kinase that associates with the Na^+K^+ATPase responsible for sodium transport and maintaining cell volume. Function or overexpression of SIK1 has not previously been associated with growth promotion. Hence, in the context of MDS, this may represent a novel gene that modulates cell growth control and differentiation. SF3B1 and TET2 are particularly exciting candidates to emerge from this screen, since mutation of both of these genes has been observed in a significant fraction of MDS patients. The clones isolated containing SF3B1 and TET2 encoded only a fraction of the cDNA in both cases (64% and 41%, respectively), representing a significant fraction of the 5' ends. Thus, in both of these cases, complementation with truncated wild type SF3B1 and TET2 cDNA had the effect of permitting CFU formation in vitro and chimeric engraftment in vivo. Ongoing work with these two isolates is aimed at full length expression of these proteins to determine whether CFU formation is enhanced by the holo-protein in MDS cells, or whether stimulation of CFU formation is restricted only to the truncated variants.

We have identified eight cDNAs that when expressed singly in human MDS bone marrow cells, have been able to confer a phenotype in which transfected MDS clones exhibited gain of function and regained the ability to differentiate into functional myeloid cells and supported successful engraftment of SG3 mice. Identification of some of these cDNAs that can successfully complement the MDS phenotype can be potentially investigated in future human gene therapy trials. Isolation of SIK1 suggests that there are alternate pathways that when activated, can confer a normal differentiation phenotype in myeloid cells. Due to the unbiased, completely random nature of this complementation technique, and the underlying abnormal biochemical pathways at work in the MDS clones, it is unlikely that simple complementation with a single cDNA will correct a majority of MDS defects and allow normal differentiation to proceed in patients. We never observed circulating human CD45 cells in peripheral blood, suggesting that SG3 mice, while and improvement over the NSG strain, is still not an ideal model system for study of MDS. However, the successful long term engraftment (6 mo) of transfected MDS cells, compared to the inability of untransfected cells to engraft, suggest that expression of these cDNAs in trans is sufficient to enhance cell survival and differentiation in the marrow compartment.

Personnel Receiving Pay from the Research Effort:

Daniel J. Lindner, M.D., Ph.D.

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Appendix

Protein sequence data for eight isolated clones

HRAS
"MTEYKLVVVGAGGVGKSALTIQLIQNHFVDEYDPTIEDSYRKQVVIDGETCLLDILDTAGQEEYSAMRDQYMRTGEGFLCVFAINNTK
SFEDIHQYREQIKRVKDSDDVPMVLVGNKCDLAARTVESRQAQDLARSYGIPYIETSAKTRQGVEDAFYTLVREIRQHKLRKLNPPDES
GPGCMSCKCVLS"

CDC25C
"MSTELFSSTREEGSSGSGPSFRSNQRKMLNLLLERDTSFTVCPDVPRTPVGKFLGDSANLSILSGGTPKRCLDLSNLSSGEITATQLT
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LRCRSQSKVQEGERQLREQIALLVKDMSP"

MYC
"MPLNVSFTNRNYDLDYDSVQPYFYCDEEENFYQQQQQSELQPPAPSEDIWKKFELLPTPPLSPSRRSGLCSPSYVAVTPFSLRGDNDG
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RRTHNVLERQRRNELKRSFFALRDQIPELENNEKAPKVVILKKATAYILSVQAEEQKLISEEDLLRKRREQLKHKLEQLRNSCA"

MAP3K7
"MSTASAASSSSSSSAGEMIEAPSQVLNFEEIDYKEIEVEEVVGRGAFGVVCKAKWRAKDVAIKQIESESERKAFIVELRQLSRVNHPN
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MAP3K8
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YIDLGALAGYFNLVRGSPTLEYG"

SF3B1 (clone that we isolated)
"MAKIAKTHEDIEAQIREIQGKKAALDEAQGVGLDSTGYYDQEIYGGSDSRFAGYVTSIAATELEDDDDDYSSSTSLLGQKKPGYHAPV
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YVRNTTARAFAVVASALGIPSLLPFLKAVCKSKKSWQARHTGIKIVQQIAILMGCAILPHLRSLVEIIEHGLVDEQQKVRTISALAIAA
LAEAATPYGIESFDSVLKPLWKGIRQHRGKGLAAFLKAIGYLIPLMDAEYANYYTREVMLILIREFQSPDEEMKKIVLKVVKQCCGTDG
VEANYIKTEILPPFFKHFWQHRMALDRRNYRQL”

SF3B1 (full length NCBI Reference Sequence: NM_012433.2)
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HMQTEDRTMKSVNDQPSGNLPFLKPDDIQYFDKLLVDVDESTLSPEEQKERKIMKLLLKIKNGTPPMRKAALRQITDKAREFGAGPLFN
QILPLLMSPTLEDQERHLLVKVIDRILYKLDDLVRPYVHKILVVIEPLLIDEDYYARVEGREIISNLAKAAGLATMISTMRPDIDNMDE
YVRNTTARAFAVVASALGIPSLLPFLKAVCKSKKSWQARHTGIKIVQQIAILMGCAILPHLRSLVEIIEHGLVDEQQKVRTISALAIAA
LAEAATPYGIESFDSVLKPLWKGIRQHRGKGLAAFLKAIGYLIPLMDAEYANYYTREVMLILIREFQSPDEEMKKIVLKVVKQCCGTDG
VEANYIKTEILPPFFKHFWQHRMALDRRNYRQLVDTTVELANKVGAAEIISRIVDDLKDEAEQYRKMVMETIEKIMGNLGAADIDHKLE
EQLIDGILYAFQEQTTEDSVMLNGFGTVVNALGKRVKPYLPQICGTVLWRLNNKSAKVRQQAADLISRTAVVMKTCQEEKLMGHLGVVL
YEYLGEEYPEVLGSILGALKAIVNVIGMHKMTPPIKDLLPRLTPILKNRHEKVQENCIDLVGRIADRGAEYVSAREWMRICFELLELLK
AHKKAIRRATVNTFGYIAKAIGPHDVLATLLNNLKVQERQNRVCTTVAIAIVAETCSPFTVLPALMNEYRVPELNVQNGVLKSLSFLFE
YIGEMGKDYIYAVTPLLEDALMDRDLVHRQTASAVVQHMSLGVYGFGCEDSLNHLLNYVWPNVFETSPHVIQAVMGALEGLRVAIGPCR
MLQYCLQGLFHPARKVRDVYWKIYNSIYIGSQDALIAHYPRIYNDDKNTYIRYELDYIL"

SIK1

"MVIMSEFSADPAGQGQGQQKPLRVGFYDIERTLGKGNFAVVKLARHRVTKTQVAIKIIDKTRLDSSNLEKIYREVQLMKLLNHPHIIK
LYQVMETKDMLYIVTEFAKNGEMFDYLTSNGHLSENEARKKFWQILSAVEYCHDHHIVHRDLKTENLLLDGNMDIKLADFGFGNFYKSG
EPLSTWCGSPPYAAPEVFEGKEYEGPQLDIWSLGVVLYVLVCGSLPFDGPNLPTLRQRVLEGRFRIPFFMSQDCESLIRRMLVVDPARR
ITIAQIRQHRWMRAEPCLPGPACPAFSAHSYTSNLGDYDEQALGIMQTLGVDRQRTVESLQNSSYNHFAAIYYLLLERLKEYRNAQCAR
PGPARQPRPRSSDLSGLEVPQEGLSTDPFRPALLCPQPQTLVQSVLQAEMDCELQSSLQWPLFFPVDASCSGVFRPRPVSPSSLLDTAI
SEEARQGPGLEEEQDTQESLPSSTGRRHTLAEVSTRLSPLTAPCIVVSPSTTASPAEGTSSDSCLTFSASKSPAGLSGTPATQGLLGAC
SPVRLASPFLGSQSATPVLQAQGGLGGAVLLPVSFQEGRRASDTSLTQGLKAFRQQLRKTTRTKGFLGLNKIKGLARQVCQAPASRASR
GGLSPFHAPAQSPGLHGGAAGSREGWSLLEEVLEQQRLLQLQHHPAAAPGCSQAPQPAPAPFVIAPCDGPGAAPLPSTLLTSGLPLLPP
PLLQTGASPVASAAQLLDTHLHIGTGPTALPAVPPPRLARLAPGCEPLGLLQGDCEMEDLMPCSLGTFVLVQ"

TET2 (clone that we isolated)
"MEQDRTNHVEGNRLSPFLIPSPPICQTEPLATKLQNGSPLPERAHPEVNGDTKWHSFKSYYGIPCMKGSQNSRVSPDFTQESRGYSKC
LQNGGIKRTVSEPSLSGLLQIKKLKQDQKANGERRNFGVSQERNPGESSQPNVSDLSDKKESVSSVAQENAVKDFTSFSTHNCSGPENP
ELQILNEQEGKSANYHDKNIVLLKNKAVLMPNGATVSASSVEHTHGELLEKTLSQYYPDCVSIAVQKTTSHINAINSQATNELSCEITH
PSHTSGQINSAQTSNSELPPKPAAVVSEACDADDADNASKLAAMLNTCSFQKPEQLQQQKSVFEICPSPAENNIQGTTKLASGEEFCSG
SSSNLQAPGGSSERYLKQNEMNGAYFKQSSVFTKDSFSATTTPPPPSQLLLSPPPPLPQVPQLPSEGKSTLNGGVLEEHHHYPNQSNTT
LLREVKIEGKPEAPPSQSPNPSTHVCSPSPMLSERPQNNCVNRNDIQTAGTMTVPLCSEKTRPMSEHLKHNPPIFGSSGELQDNCQQLM
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KTTQLEHKSQMYQVEMNQGQSQGTVDQHLQFQKPSHQVHFSKTDHLPKAHVQSLCGTRFHFQQRADSQTEKLMSPVLKQHLNQQASETE
PFSNSHLLQHKPHKQAAQTQPSQSSHLPQNQQQQQKLQIKNKEEILQTFPHPQSNNDQQREGSFFGQTKVEECFHGENQYSKSSEFETH
NVQMGLEEVQNINR”

TET2 (full length NCBI Reference Sequence: NM_001127208.2
"MEQDRTNHVEGNRLSPFLIPSPPICQTEPLATKLQNGSPLPERAHPEVNGDTKWHSFKSYYGIPCMKGSQNSRVSPDFTQESRGYSKC
LQNGGIKRTVSEPSLSGLLQIKKLKQDQKANGERRNFGVSQERNPGESSQPNVSDLSDKKESVSSVAQENAVKDFTSFSTHNCSGPENP
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PSHTSGQINSAQTSNSELPPKPAAVVSEACDADDADNASKLAAMLNTCSFQKPEQLQQQKSVFEICPSPAENNIQGTTKLASGEEFCSG
SSSNLQAPGGSSERYLKQNEMNGAYFKQSSVFTKDSFSATTTPPPPSQLLLSPPPPLPQVPQLPSEGKSTLNGGVLEEHHHYPNQSNTT
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RNKEQEILKGRDKEQTRDLVPPTQHYLKPGWIELKAPRFHQAESHLKRNEASLPSILQYQPNLSNQMTSKQYTGNSNMPGGLPRQAYTQ
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PFSNSHLLQHKPHKQAAQTQPSQSSHLPQNQQQQQKLQIKNKEEILQTFPHPQSNNDQQREGSFFGQTKVEECFHGENQYSKSSEFETH
NVQMGLEEVQNINRRNSPYSQTMKSSACKIQVSCSNNTHLVSENKEQTTHPELFAGNKTQNLHHMQYFPNNVIPKQDLLHRCFQEQEQK
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ELLRLSGPVMQQSQQPQPLQKQPPQPQQQQRPQQQQPHHPQTESVNSYSASGSTNPYMRRPNPVSPYPNSSHTSDIYGSTSPMNFYSTS
SQAAGSYLNSSNPMNPYPGLLNQNTQYPSYQCNGNLSVDNCSPYLGSYSPQSQPMDLYRYPSQDPLSKLSLPPIHTLYQPRFGNSQSFT
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LHLQNKENDMLSHTANGLSKMLPALNHDRTACVQGGLHKLSDANGQEKQPLALVQGVASGAEDNDEVWSDSEQSFLDPDIGGVAVAPTH
GSILIECAKRELHATTPLKNPNRNHPTRISLVFYQHKSMNEPKHGLALWEAKMAEKAREKEEECEKYGPDYVPQKSHGKKVKREPAEPH
ETSEPTYLRFIKSLAERTMSVTTDSTVTTSPYAFTRVTGPYNRYI"