SUPPLEMENTARY INFORMATION

Identification of additional IDH mutations associated with oncometabolite *R*(-)-2-hydroxyglutarate production

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Supplementary Materials and Methods

Patient Selection and Sequence Analysis of IDH1 and IDH2

DNA was isolated from peripheral blood and/or bone marrow from a total of 976 patients with hematologic malignancies including 553 acute myeloid leukemia, 322 myeloproliferative neoplasm, 54 myelodysplastic syndrome, 44 chronic myelomonocytic leukemia, and 3 T-cell angioimmunoblastic lymphoma (AILT) patients. All patients provided authorization for use of their medical records for research purposes. Approval was obtained from institutional review boards at Memorial Sloan-Kettering or Dana-Farber, and informed consent was provided according to the Declaration of Helsinki. DNA resequencing of all IDH1 residues between amino acid residues 41–138 as well as all IDH2 residues between amino acids 125-226 were performed in all patients. Sequencing of the entire coding region of IDH1 and IDH2 was also performed in 20 patients with AML and in 3 patients with AILT. Sequence analysis was performed using Mutation Surveyor (SoftGenetics, State College, PA, USA) and all mutations were validated by repeat PCR and sequencing on unamplified DNA from the archival sample.

Constructing IDH1 and IDH2 Mutants

The human IDH1 cDNA clone BC012846.1 was purchased from ATCC in pCMV-sport6, and human IDH2 (BC009244) was purchased from Invitrogen in pOTB7. Standard site-directed mutagenesis techniques were used to generate point mutations. Wild-type and mutant sequences were subcloned into pcDNA3 or LPC vectors before expression in cells. All plasmids were tested for integrity by restriction digest, and for each construct direct sequencing of the entire coding region and flanking sequence was performed to confirm only the desired mutation was introduced.

Cell Culture and Transfection

293T cells were cultured in DMEM (Dulbecco's modified Eagles's medium; Invitrogen) with 10% fetal bovine serum (CellGro). Cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Structural and Energetic Modeling

For homology modeling of IDH1 mutants, using the structure PDB ID 1T0L as the template, the appropriate residue was mutated using the program EVOLVE (Lewis and Sharp 2011), and the residue side chain was then repacked by searching rotamer space followed by minimization of the mutated residue and neighboring residues within 10Å using a torsional minimizer to generate the minimum energy structure. The CHARMM27 energy function was used for model building (MacKerell *et al.,* 1998). Structures were visualized, and atomic distances computed using PyMol. Electrostatic interactions of active site residues with the β-carboxylate residue of isocitrate were calculated from the continuum electrostatic Poisson-Boltzmann model using the program DelPhi (Gilson *et al.,* 1998; Sharp 1998), with a solvent dielectric of 80, a protein dielectric of 4, and an ionic strength of 0.15M. Partial charges were the same used for the CHARMM27 potential in model building.

Supplementary References

Gilson M, Sharp KA, Honig B. (1998). Calculating the Electrostatic Potential of Molecules in Solution: Method and Error Assessment. *J Comp Chem* **9:** 327-335.

Lewis M, Sharp KA (2011). EVOLVE: A computational tool for in silico screening and modeling of protein-DNA complexes.

MacKerell AD, Brooks B, Brooks CL, Nilsson L, Roux B, Won Y *et al.* (1998). CHARMM: The Energy Function and its Parameterization with an Overview of the Program. In: Schleyer PvR (ed). *The Encyclopedia of Computational Chemistry*. John Wiley & Sons: Chichester, UK. pp 271-277.

Sharp KA. (1998). Calculation of HyHel10-lysozyme binding free energy changes: effect of ten point mutations. *Proteins* **33:** 39-48.

Supplementary Figure Legends

Supp. Figure 1. Common IDH SNPs and rare, single-sample, IDH alterations do not produce 2HG and retain the wild-type ability to increase NADPH production with isocitrate.

(A) Western blot confirming expression of IDH SNPs and singly-described variants 48 h following transfection in 293T cells, with both wild-type and the known 2HG-producing mutants IDH1 R132H and IDH2 R140Q included as controls.

(B) Cells transfected in parallel with (A) were extracted 48 h post-transfection for intracellular metabolites which were then MTBSTFA-derivatized and analyzed by GC-MS. Shown is the quantitation of 2HG signal intensity relative to glutamate.

(C) Lysates from (A) were assayed for their ability to produce NADPH from $NADP^+$ in the presence of 0.1 mM isocitrate (using 3 µg lysate protein for IDH1 assays and 1 µg lysate protein for IDH2 assays). Data are presented as the mean and SD from three independent measurements at the indicated time points. Data for (A-C) are from a representative of two independent experiments.

Supp. Figure 2. Rare F394 mutants of IDH2 do not elevate 2HG levels in transfected cells.

(A) F394I and F394V mutants of IDH2 were transfected in 293T cells, along with empty vector, IDH2 wild-type, and 2HG-producing mutants IDH2 R172K and R140Q as controls. Cells were lysed 48 h after transfection and assessed for IDH2 protein expression with goat polyclonal antibody (Santa Cruz, sc55666) and confirmed with mouse monoclonal antibody (Abcam, ab55271; data not shown). S6 expression was used as a loading control. Data are from a representative of five independent experiments.

(B) Overexpression of IDH1/2 mRNA was confirmed by isolating RNA from cells transfected in parallel to (A). RNA was extracted with Trizol (Invitrogen) and, following DNAse digestion, cDNA was synthesized with Superscript II reverse transcriptase (Invitrogen). Quantitative PCR was performed on a 7900HT sequence detection system using Taqman Gene Expression Assays (Applied Biosystems). IDH2 was measured with probe Hs00158033_m1. Data are normalized to actin mRNA, quantified relative to vector-transfected cells, and presented as the mean and SD of three measurements. Elevated IDH2 mRNA levels in transfected cells were confirmed with an additional probe Hs00953884_g1 (data not shown).

(C) 293T cells were transfected and extracted 48 h later for intracellular metabolites which were then MTBSTFA-derivatized and analyzed by GC-MS. Quantitation of 2HG signal intensity relative to glutamate from a representative of three independent experiments is shown.

(D) Cell lysates were assayed for their ability to produce NADPH from $NADP^+$ in the presence of 0.1 mM isocitrate. Data are presented as the mean and SD from three

independent measurements at the indicated time points and are from a representative of two independent experiments.

Supp. Figure 3. Rare IDH1 mutants G70D, A134D, and R49C do not elevate 2HG levels in transfected cells.

(A) IDH1 G70D, A134D, and R49C mutants were transfected in 293T cells, along with empty vector, IDH1 WT, and 2HG-producing IDH1 R132H as controls. Cells were lysed 48 h after transfection and assessed for IDH1 protein expression, with S6 expression as a loading control. Data are from a representative of two independent experiments.

(B) RNA from cells transfected in parallel to (A) was extracted 48 h following transfection and analyzed by RT-qPCR for expression of IDH1 mRNA (Taqman probe Hs00271858_m1) relative to actin. Quantitation is shown relative to levels in vector-transfected cells. Data are presented as the mean and SD of three measurements.

(C) Cells transfected in parallel to (A) were extracted for intracellular metabolites which were derivatized with MTBSTFA and analyzed by GC-MS. Shown is the quantitation of 2HG signal intensity relative to glutamate from a representative of two independent experiments.

(D) Lysates from (A) were assayed for their ability to convert $NADP^+$ to NADPH in the presence of 0.1 mM isocitrate. Data are presented as the mean and SD from three independent measurements at the indicated time points and are from a representative of three independent experiments.

Supp. Figure 4. Expression of A134D mutant IDH1 does not decrease isocitrate-dependent NADPH production from either overexpressed wild-type IDH1 or endogenous $NADP^+$-IDH activity.

(A) 293T cells were transfected with the listed amounts of wild-type and/or A134D mutant IDH1, or empty vector. Dose-dependent expression levels of IDH1 protein were confirmed by Western blot.

(B) Lysates from (A) were assayed for their ability to generate NADPH from $NADP^+$ in the presence of 0.1 mM isocitrate with 10 µg lysate protein. Data are presented as the mean and standard deviation of three independent measurements at the indicated time points and are from a representative of two independent experiments.

Supp. Figure 5. IDH1 G70D, IDH1 R49C, IDH2 F394I, and IDH2 F394V mutants can be transcribed and translated *in vitro.*

(A) IDH1 WT, IDH1 G70D, and IDH1 R49C cDNAs, which were encoded in the pCMV-Sport6 vector with an upstream SP6 promoter were transcribed and translated *in vitro* with a TnT SP6 coupled reticulocyte lysate system (Promega) according to the manufacturer's instructions. Briefly, plasmids were incubated with SP6 RNA polymerase, reticulocyte lysate, and 0.04 mCi of [^{35}S]methionine (>1000 Ci/mmol; PerkinElmer) at 30°C for 90 min. The translated products were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gel was treated with Amplify Reagent (GE Healthcare) prior to drying and fluorographic detection. A reaction with no DNA template was included as a negative control, while a reaction with a construct

encoding luciferase (61 kD), provided by the manufacturer, was included as a positive control.

(B) IDH2 WT, IDH2 F394I, and IDH2 F394V cDNAs, which were encoded in the pCDNA3 vector with an upstream T7 promoter, were transcribed and translated *in vitro* with a TnT T7 coupled reticulocyte lysate system. Plasmids were incubated with T7 RNA polymerase, reticulocyte lysate, and 0.04 mCi of [^{35}S]methionine (>1000 Ci/mmol) at 30°C for 90 min. Translation products were subsequently analyzed as in (A). A reaction with no DNA template was included as a negative control, while a reaction with a construct encoding luciferase (61 kD) was included as a positive control. Data for (A) and (B) are from a representative of two independent experiments.

Supp. Table 1. Key distances (Å) from IDH1 active site residues to isocitrate's β-carboxyl in IDH1 WT and R132H, R100A, G97D, and Y139D mutants.

IDH1 Residue	WT	R132H	R100A	G97D	Y139D
132	3.1	5.6	2.8	2.9	3.8
100	2.8	2.7	7.5	3.0	2.8
97	5.9	5.9	5.9	3.6	5.9
139	3.8	3.8	4.0	4.0	3.9

Similar to the IDH2 R140 mutation studied previously and to IDH1 R132H mutation, mutation of IDH1 R100A is predicted to increase the distance from residue 100 to the nearest neighboring atom on isocitrate's β-carboxyl. However, for the IDH1 G97D and Y139D mutations, the distance between the affected residue and isocitrate's β-carboxyl is not greatly increased compared to IDH1 WT, consistent with these mutants having an alternative mechanism of charge repulsion, rather than reduced proximity of a stabilizing interaction, that can contribute to favoring 2HG production over isocitrate utilization.

Supplementary Table 2

PubMed search (terms "IDH1 OR IDH2") of unique cases of IDH mutation reported in literature (as of June 30th, 2011)
130 publications total, plus unpublished mutation in COSMIC database and novel IDH2 F394I/V mutations described in the current manuscript.
Counts do not include synonymous/silent mutations and known SNPs IDH1 V71I and V178I.
All IDH mutations are at IDH1 R132, IDH2 R172, or IDH2 R140 (previously established 2HG-producing alleles) unless otherwise noted.

Study	tumor type	Tumors with IDH1 mutation	Tumors with IDH2 mutation	Notes
Sjoblom *et al.,* 2006	colon CA	1		
Parsons *et al* ., 2008	glioma	18		
Balss *et al.,* 2008	glioma	221		
Bleeker *et al.,* 2009	glioma/colon CA cell lines	25		two G97D cases, in two colon CA cell lines
Yan *et al.,* 2009	glioma	161	9	
De Carli *et al.,* 2009	glioma	159		
Durcay *et al.,* 2009	glioma	40		
Watanabe *et al.,* 2009, Am J Pathol	glioma	130		
Watanabe *et al.,* 2009, Acta Neuropath	glioma/Li-Frauemeni	5		
Zhao *et al.,* 2009	glioma	8		
Kang *et al.,* 2009	glioma/prostate/B-ALL	7		
Hayden *et al.,* 2009	adult sPNET	2		
Ichimura *et al.,* 2009	glioma	119		
Korshunov *et al.,* 2009	glioma	38		
Hartmann *et al.,* 2009	glioma	716	31	
Sanson *et al.,* 2009	glioma	155		
Mardis *et al.,* 2009	AML	16		
Nobusawa *et al.,* 2009	glioma	36		
Sonoda *et al.,* 2009	glioma	39	1	
Weller *et al.,* 2009	glioma	16		
Gaal *et al.,* 2010	paraganglioma	1		
Horbinski *et al.,* 2009	glioma/ganglioma	36	1	
Gravendeel *et al.,* 2009	glioma	83		
Dubbink *et al.,* 2009	glioma	42		
Dang *et al.,* 2009	glioma	12		
Abdel-Wahab *et al.,* 2010	sAML	5		
Gravendeel *et al.,* 2010	glioma	246		
Chou *et al.,* 2010	AML	27		
Waha *et al.,* 2010	glioma	29	2	
Felsberg *et al.,* 2010	glioma	63	6	
Gross *et al.,* 2010	AML	11	2	
Dougherty *et al.,* 2010	glioma	1		
Green and Beer, 2010, NEJM	AML	3	2	
van den Bent *et al.,* 2010	glioma (oligos)	73	1	
Ward *et al.,* 2010	AML	10	17	
Murugan *et al.,* 2010	thyroid	1		one G123R mutant sample, homozygous, no analysis of paired normal tissue
Andrulis *et al.,* 2010, Leuk Res	MDS	3		
Wagner *et al.,* 2010	AML	30		
Marcucci *et al.,* 2010	AML	49	69	
Kosmider *et al.,* 2010	MDS/MPN	4	13	
Ho *et al.,* 2010	AML	12		
Noushmehr *et al.,* 2010	glioma	18		
Pardanani *et al.,* 2010	MPN	5	4	
Thol *et al.,* 2010	AML		33	
Labussiere *et al.,* 2010	glioma	315	16	
Horbinksi *et al.,* 2010	glioma	42	3	
Toedt *et al.,* 2010	glioma	44	1	
Paugh *et al.,* 2010	pediatric GBM	2		one G97D, one R49C. Confirmed somatic. Also found focal homozygous IDH1 deletion
Pardanani *et al.* , 2010 (Jul), Leukemia	MDS/AML	6		
Thol *et al.,* 2010, Hematologica	MDS/AML	11	4	
Andrulis *et al.,* 2010, Hematologica	MPN	3		

Tefferi *et al.,* 2010	MPN	18	20	
Seiz *et al.,* 2010	glioma	10		
McDonald *et al.,* 2010	glioma	11		
Abbas *et al.,* 2010	AML	55	97	
Bujko *et al.,* 2010	glioma	8		
Paschka *et al.,* 2010	AML	61	70	
Lopez *et al.,* 2010	melanoma metastasis	1		
Boissel *et al.,* 2010	glioma	50	15	
Green *et al.,* 2010 (Jul) Blood	AML	107		
Sellner *et al.,* 2010	AML	3	1	
Capper *et al.,* 2010	glioma	13		
Rocquain *et al.,* 2010	MDS/AML	5	18	
Hemerly *et al.,* 2010	thyroid	10		six cases G70D, two cases A134D, one case I130M, one case H133Q. Absent in paired normal thyroid
Pardanani *et al.,* 2010 (Oct), Leukemia	MPN		1	
Pollack *et al.,* 2011	pediatric malignant glioma	7		
Schnittget *et al.,* 2010	AML	93		
Ferrer-Luna *et al.,* 2010	glioma (oligos)	22		
Setty *et al.,* 2010	pediatric glioma	11		
Caramazza *et al.,* 2010	AML/MDS/lymphoma	3	15	
Pusch *et al.,* 2011	glioma	3		three IDH1 R100Q cases
Laffaire *et al.,* 2011	glioma	53		
Pigazzi *et al.,* 2011	pediatric AML	4		
Jeziskova *et al.,* 2010	AML		4	
Zou *et al.,* 2010	AML, NHL	5	4	Includes one IDH1 I99M. Not confirmed to be somatic. Occurs in same patient with IDH2 R140Q.
Noordermer *et al.,* 2011	AML	15	17	
Yoshida *et al.,* 2011	AML	9	7	Includes one case IDH1 R100X (nonsense). Not confirmed to be somatic.
Soverini *et al.,* 2011	CML		4	
Houillier *et al.,* 2010	glioma	125	7	
Motomura *et al.,* 2010	glioma	4		
Capper *et al.,* 2011	glioma, neurocytic lesion	158	5	
Kim *et al.,* 2011	glioma	305	10	
Chou *et al.,* 2011	AML	27	54	
Metellus *et al.,* 2011	glioma	38	2	
Hartmann *et al.,* 2010	glioma	211	1	
Miwa *et al.,* 2011	pediatric glioma	2		
Jha *et al.,* 2010	glioma	40		
Figueroa *et al.,* 2010	AML	24	33	
Christensen *et al.,* 2011	glioma	56	2	
Patel *et al.,* 2011	AML	12	4	
Taal *et al.,* 2011	glioma	36	0	
Ducray *et al.,* 2011	glioma	198		
Williams *et al.,* 2011	glioma	25		
Ikota *et al.,* 2011	glioma and other brain tumor	48		
de Tayrac *et al.,* 2011	glioma	30		
Oki *et al.,* 2011	pediatric AML	0	1	
Wagner *et al.,* 2011	AML	11	11	
Mokhtari *et al.,* 2011	glioma	64	9	
Pichler *et al.,* 2011	t-AML, s-AML	1	6	one IDH2 R140Q--> uniparental disomy
Xu *et al.,* 2011	glioma	10		
Kunz *et al.,* 2011	glioma	9		
Horbinski *et al.,* 2011	gangliogliomas	8	2	Both IDH2 mutations are nonsense
Piaskowski *et al.,* 2011	glioma	17		
Jones *et al.,* 2011	glioma	13	2	
Camelo-Piragua *et al.,* 2011	glioma	14	1	
Takano *et al.,* 2011	glioma	12		
Makishima *et al.,* 2011	CML blast phase	1	1	
Shibata *et al.,* 2011	melanoma	2	2	IDH2 mutations are G171D and P158T
Damm *et al.,* 2011	AML	33	36	

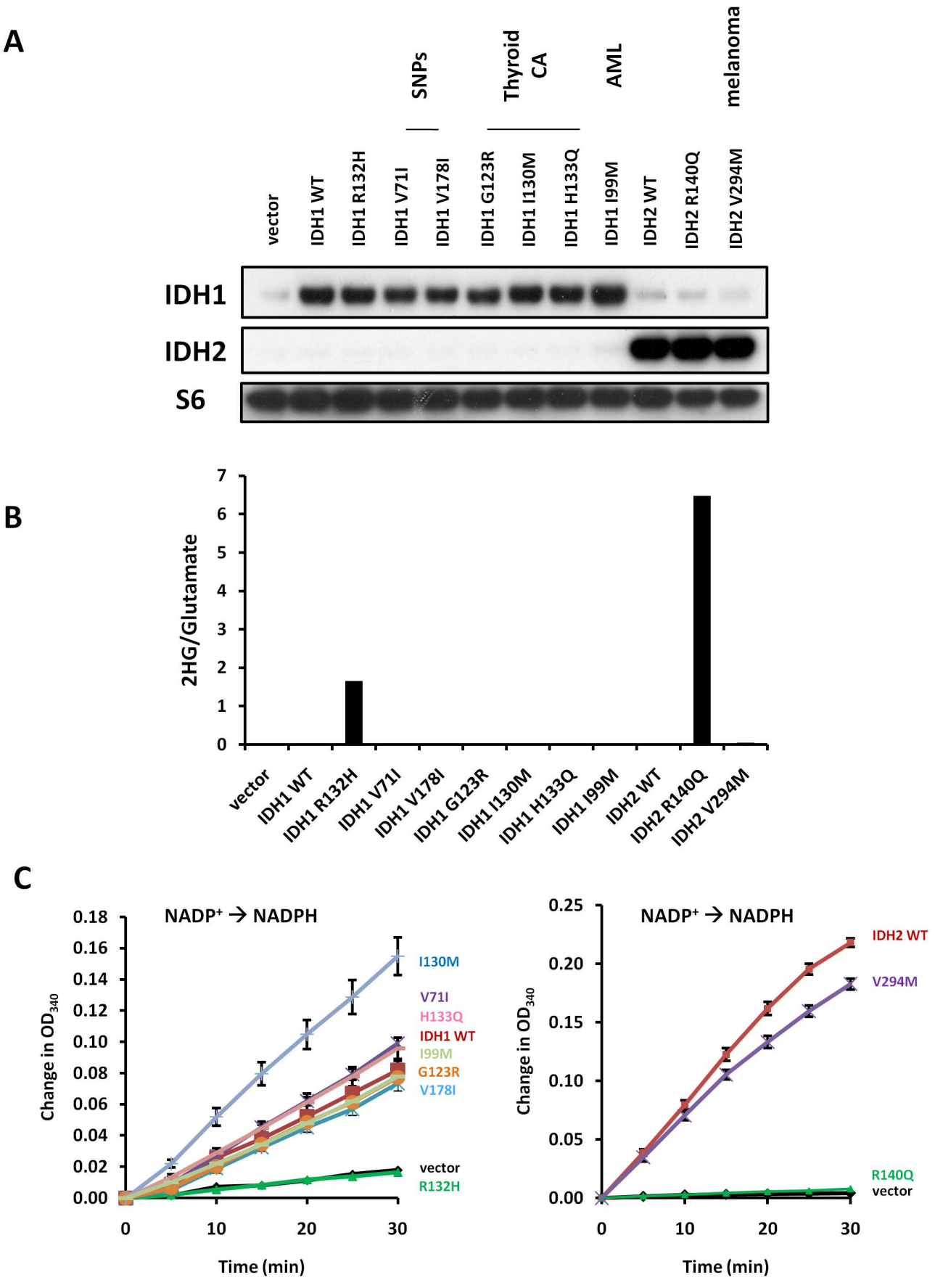
Grauer *et al.,* 2011	glioma	48		
Gessi *et al.,* 2011	s-PNET	2		
Uno *et al.,* 2011	glioblastoma	19		
Boissel *et al.,* 2011	AML		18	
Motomura *et al.,* 2011	glioblastoma	4	0	
Narasimhaiah *et al.,* 2011	gliomatosis cerebri	5		
Saetta *et al.,* 2011	glioma	15		
Ho *et al.,* 2011	pediatric AML		4	
Desestret *et al.,* 2011	gliomatosis cerebri	17		
Hartmann *et al.,* 2011	glioma	92		
Jha *et al.,* 2011	glioma	46		
Thol, Friesen *et al.,* 2011	MDS	6		
Rockova *et al.,* 2011	AML	32	36	
Green *et al.,* 2011	AML		148	
Amary *et al.,* 2011	cartilaginous tumors	74	7	
Krell *et al.,* 2011	glioblastoma	6		
Mellai *et al.,* 2011	glioma	64		
Metellus *et al.,* 2011	glioma	20		
Damm *et al.,* 2011	pediatric AML	8	10	
Andersson *et al.,* 2011	pediatric ALL/AML	4	5	
Thol, Damm *et al.,* 2011	AML	35	35	
COSMIC database, no publication	melanoma	1		IDH2 V294M
found in this study	AILT		2	IDH2 F394I and F394V
		Tumors with IDH1 mutation	**Tumors with IDH2 mutation**	**IDH1/2 combined**
	TOTAL	**5708**	**942**	**6650**

6624 out of 6650 cases have IDH1 R132, IDH2 R172,or IDH2 R140 mutant alleles previously shown to produce 2HG.

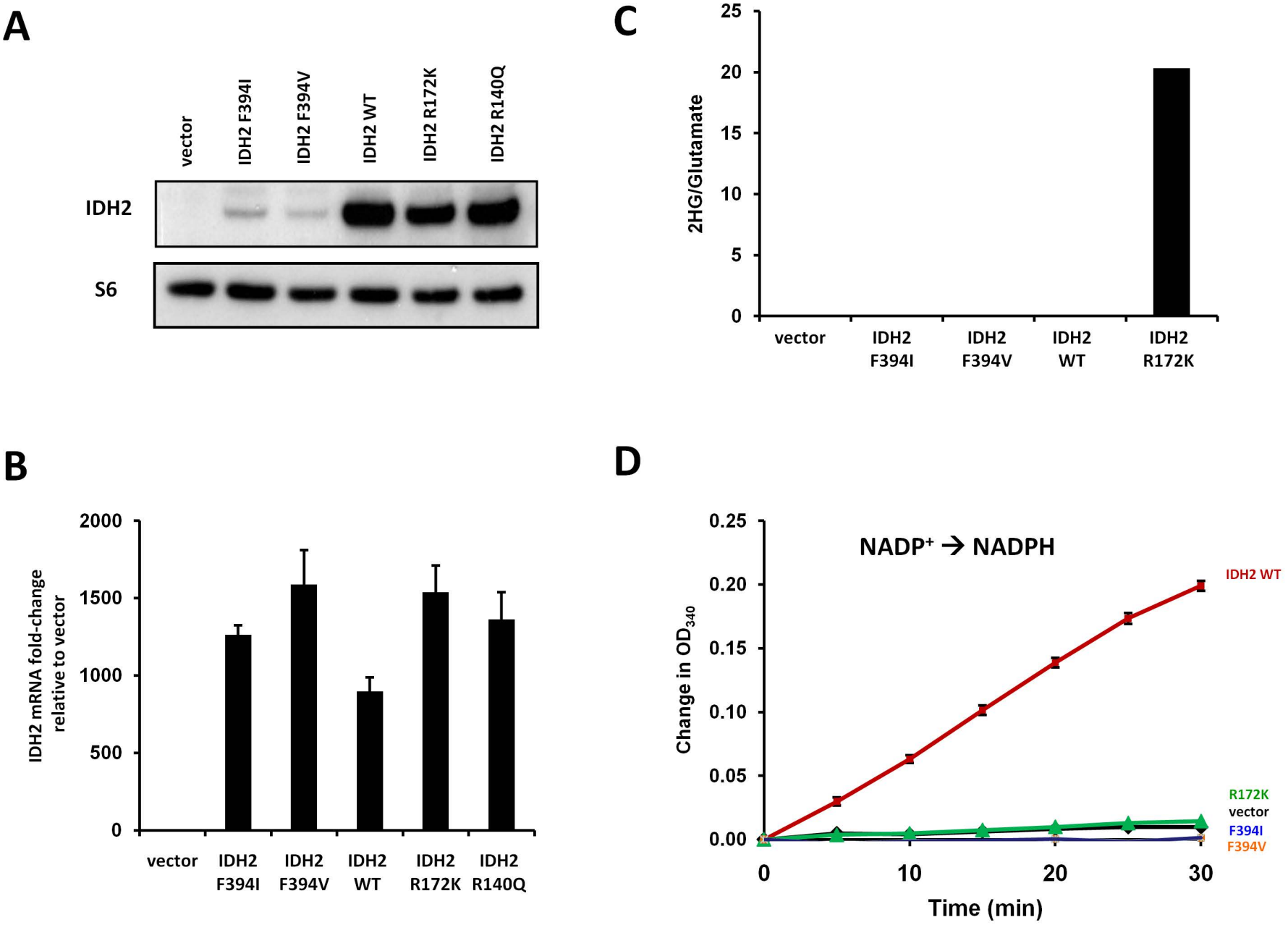
26 out of 6650 cases have mutations at residues other than IDH1 R132, IDH2 R172, and IDH2 R140:

Occurrence of new 2HG-producing alleles:			
*IDH1 G97D	2		
*IDH1 R100Q	3		
*IDH1 Y139D	0		
Total	5		
Percentage of all reported IDH mutant cases:		5 out of 6650	**0.08%**
Occurrence of alleles demonstrated to have WT activity			
*IDH1 G123R	1		
*IDH1 I130M	1		
*IDH1 H133Q	1		
*IDH1 I99M	1		
*IDH2 V294M	1		
Total	5		
Percentage of all reported IDH mutant cases:		5 out of 6650	**0.08%**
Occurrence of alleles demonstrated to have loss of expression/function			
*IDH1 G70D	6		
*IDH1 A134D	2		
*IDH1 R49C	1		
*IDH2 F394I/V	2		
*IDH1/2 nonsense	3		
Total	14		
Percentage of all reported IDH mutant cases:		14 out of 6650	**0.21%**
Additional single-sample alleles recently reported:			
*IDH2 P158T	1		
*IDH2 G171D	1		
Total	2		
Percentage of all reported IDH mutant cases:		2 out of 6650	**0.03%**

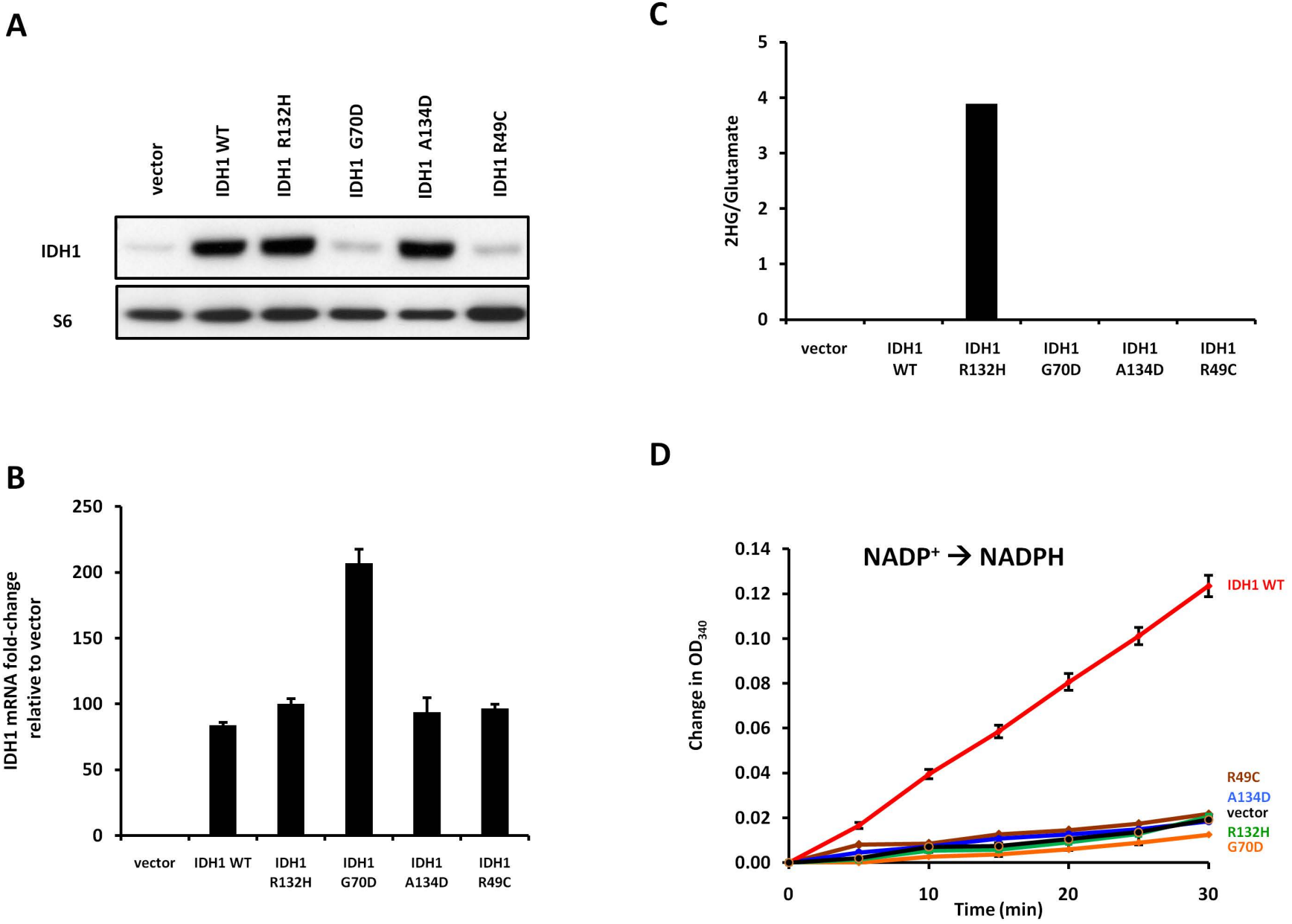
Supp. Figure 1.



Supp. Figure 2.

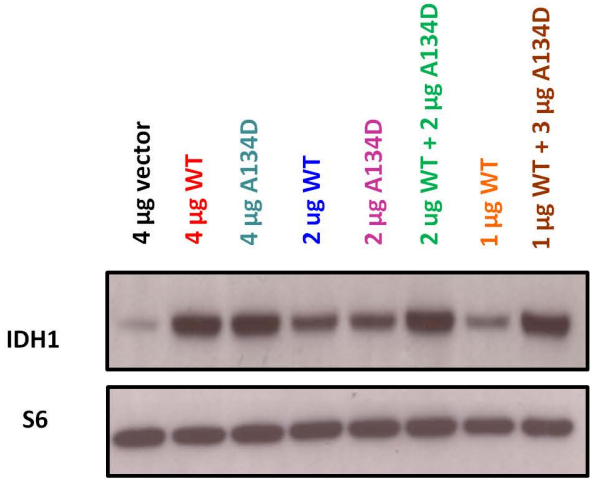


Supp Figure 3.



Supp Figure 4.

A



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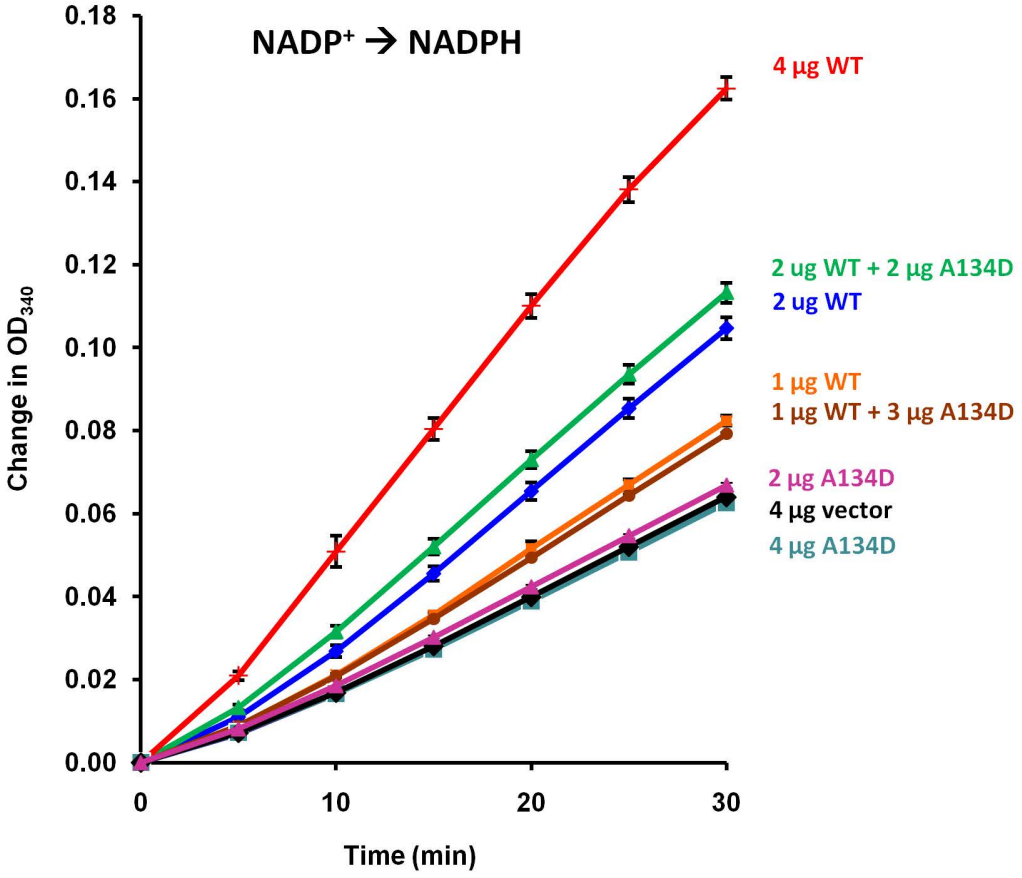

Supp Figure 5.