Evidence for a Tumor Suppressor Role for the *Large Tumor Suppressor* Genes *LATS1* and *LATS2* in Human Cancer

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ABSTRACT The role of Large tumor suppressor LATS/Warts in human cancer is not clearly understood. Here we show that *hLATS1/2* cancer mutations affect their expression and kinase activity. *hLATS1/2* mutants exhibit a decreased activity in inhibiting YAP and tissue growth. Therefore, *hLATS1/2* alleles from human cancer can be loss-of-function mutations.

LARGE tumor suppressor (Lats)/Warts (Wts) plays a critical role in mediating Hippo (Hpo) growth-inhibitory signaling but its role in human cancer is less clear (recently reviewed in Harvey *et al.* 2013; Yu and Guan 2013). In the Catalogue of Somatic Mutation in Cancer (COSMIC) database, 58 nonsynonymous *hLATS1* somatic mutations and 43 for *hLATS2* have been identified from >5000 unique human cancer samples (Supporting Information, Table S1). Therefore, *hLATS1/2* genes are mutated in 1–2% of human cancers. The primary tissue types of these mutations include lung, ovary, breast, and a number of other organs (Table S1). In this study, several computationally predicted damaging mutations have been experimentally investigated, which include hLATS1-V719I, hLATS1-R806P, hLATS2-G40E, hLATS2-G909R, and hLATS2-C953* (Table S2). V719, R806, and G909 are conserved from nematode to vertebrate, whereas G40 is conserved only in vertebrates (Figure S1).

Through Western blot analyses, we found that V719I and R806P mutations did not significantly alter their expression levels compared to wild type. However, V719I moderately and R806P dramatically reduced hLATS1 kinase activity (Figure S2). For hLATS2 (Figure S2), G40E abolished its expression as well as activity; G909R did not alter expression level but significantly reduced its activity. A nonsense mutation C953* deletes part of its kinase domain and C-terminal region. As predicted, C953* led to the production of a truncated inactive protein. Thus, these *hLATS1/2* alleles are loss-of-function mutations.

To test the activity of these *hLATS1/2* alleles in tissue growth inhibition, we generated transgenic *Drosophila* lines as an *in vivo* model. *hLATS1/2* and their variants were selectively expressed in larval wing epithelium under the control of wing-specific drivers such as *MS1096-Gal4*. As expected, overexpression of wild-type hLATS1 and hLATS2 in *Drosophila* wing resulted in 34 and 45% reduction in size, respectively (Figure 1, A, B, E, and I), indicating that human *LATS* genes are sufficient to reduce tissue growth and organ size. Interestingly, hLATS1-V719I mutation does not drastically affect hLATS1 activity, as the mutant protein can still effectively reduce the organ size (Figure 1, C and I). It appears that *Drosophila* cells were not sensitive to the structural change caused by V719I alteration. In the case of hLATS2, however, G909R and C953* mutations effectively reduced their growth-inhibitory activity of hLATS2, as the wings were much less reduced in *hLATS2-G909R* and *hLATS2-C953** transgenic flies (Figure 1, E–I). Therefore, human cancer mutations such as hLATS2-G909R and hLATS2-C953* are loss-of-function mutations with reduced growth inhibitory function.

To further investigate the growth regulatory activity of *hLATS1/2*, we used RNAi lines for *wts*, the *Drosophila* ortholog of human *LATS1/2*, to monitor activities of hLATS1/2 and variants in a *wts* loss-of-function background. Previous studies have shown that expression of *hLATS1* transgene in

doi: 10.1534/genetics.113.156372
Manuscript received August 12, 2013; accepted for publication September 3, 2013
Supporting information is available online at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156372/-/DC1.
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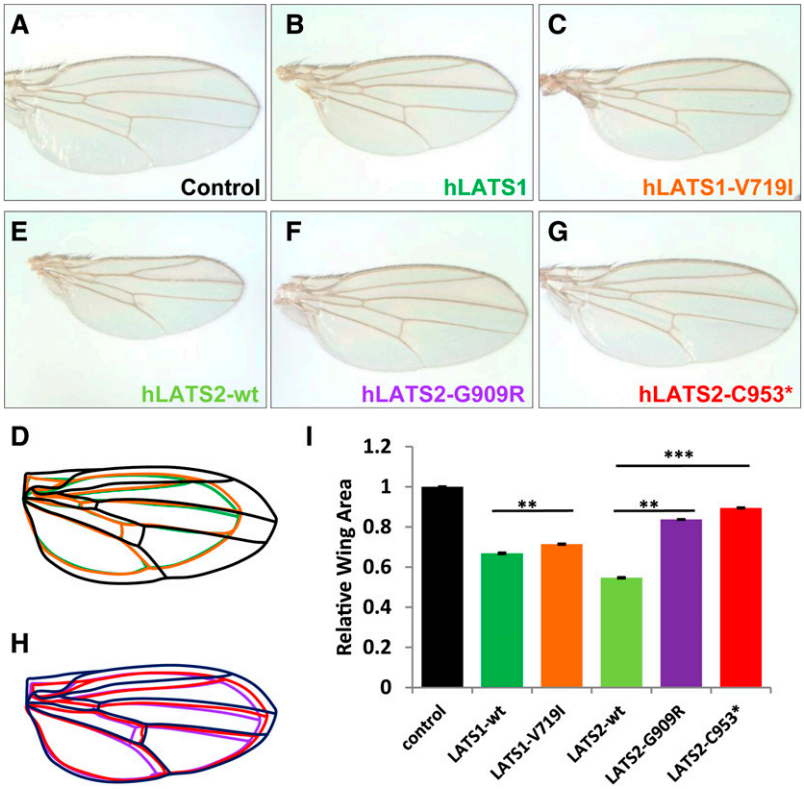

Figure 1 *In vivo* analysis of hLATS1/2 and variants for their activity to inhibit tissue growth in *Drosophila*. (A–H) Images of female wings (anterior to the top) that express following transgenes under the control of *MS1096-Gal4*. (A) *UAS-86Fb-lacZ* as a negative control. (B) *UAS-86Fb-hLATS1-wt*. (C) *UAS-86Fb-hLATS1-V719I*. (D) Outlines of the wings shown in A–C. (E) *UAS-86Fb-hLATS2-wt*. (F) *UAS-86Fb-hLATS2-G909R*. (G) *UAS-86Fb-hLATS2-C953**. (H) Outlines of the wings shown in E–G. (I) Quantification of total wing area of the genotypes (n = 40 for each genotype) displayed in A–C and E–G. Results represent mean ± SEM. $^{**}P < 10^{-11}$, $^{***}P < 10^{-53}$. Embryos from *Drosophila* attP docking strains *y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-86Fb* or *y[1] M{vas-int.Dm}ZH-2A w[*]; M{3xP3-RFP.attP}ZH-51D* (from Bloomington *Drosophila* Stock Center, BDSC; Bischof *et al.* 2007) were injected with the *pUAST-attB* constructs carrying wild-type *hLATS1/2* and mutant variants. Fly embryo injections were partly done by Rainbow Transgenic Flies. Orange eye color was a selection marker for isolating transformants. Transgenic lines were established through standard procedures. Flies with *pUAST-lacZ-attB* inserted in 51D or 86Fb (gift from Johannes Bischof) were used as controls. The *Drosophila* strains that carry *wts-RNAi* on the second chromosome (no. v106174) and the third chromosome (no. 34064) were obtained from the Vienna *Drosophila* RNAi Center (VDRC) and BDSC, respectively. The VDRC *UAS-wts-RNAi* inserted in the second chromosome was combined with *UAS-hLATS1/2* in the third chromosome (86Fb), while the BDSC *UAS-wts-RNAi* inserted in the third chromosome was combined with *UAS-hLATS1/2* at the second chromosome (51D). *MS1096-Gal4* was used to drive wing-specific expression. Adult wings were dissected and analyzed by ImageJ for size measurement.

homozygous *wts* mutants rescued lethality (Tao *et al.* 1999). Under the control of wing-specific driver *MS1096-Gal4*, *UAS-wts-RNAi* caused pupal lethality (Figure 2), which was a critical assay to monitor *in vivo* activities of wild-type hLATS1/2 and variants.

To determine the relative survival rate of strains expressing different *hLATS1/2* transgenes, we crossed $X^{MS1096\text{-}Gal4}Y$ males with transgenic *UAS-hLATS1/2* females to generate female offspring with induced *hLATS* expression, and male offspring without any *hLATS* expression, in the presence or absence of *wts-RNAi*. As controls, expression of hLATS1/2 and variants in a wild-type background caused no defects in viability (Figure 2). Compared to wild-type hLATS1, hLATS1-V719I did not fully rescue the lethality caused by *wts-RNAi* (Figure 2). Moreover, unlike wild-type hLATS2, hLATS2 mutants G909R and C953* failed to fully rescue the lethality of *wts-RNAi* flies (Figure 2). Therefore, V719I, G909R, and C953* are loss-of-function mutations and unable to fully replace the endogenous *wts* gene for normal development.

To investigate this further, the wings of female offspring were analyzed. In a *wts-RNAi* background, both hLATS1 and hLATS2 still exhibited potent growth-inhibiting activities (Figure 3, compare parts B and E with A). Compared with wild-type hLATS1, hLATS1-V719I was less capable in blocking tissue growth (Figure 3I, compare C with B). Moreover, hLATS1-R806P also appeared to be less active (Figure 3J). Interestingly, hLATS2 mutant G909R was inactive in growth inhibition as there was no longer reduction in wing size (Figure 3, E, F, and I). The G909R wing in a *wts-RNAi* background was actually 11% larger than wild-type wings, possibly

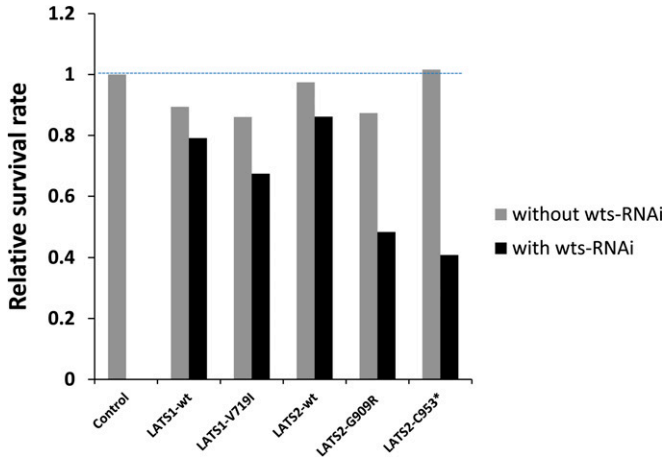

Figure 2 *In vivo* analysis of hLATS1/2 and mutants for their ability to rescue the lethality induced by loss of the endogenous *wts* gene function. Relative survival rate is measured by the number of adult females divided by the number of adult males produced by parental flies of males *MS1096-Gal4* (on the X chromosome) crossed with females with genotype as indicated. The number of offspring males serves as a control to estimate an expected number of female flies that express the *UAS* transgenes driven by *MS1096-Gal4*. In a *wts-RNAi* (II) background, the survival rate in the absence of *LATS* transgenic expression was zero (the control column). Between 40 and 148 offspring males were scored from each cross.

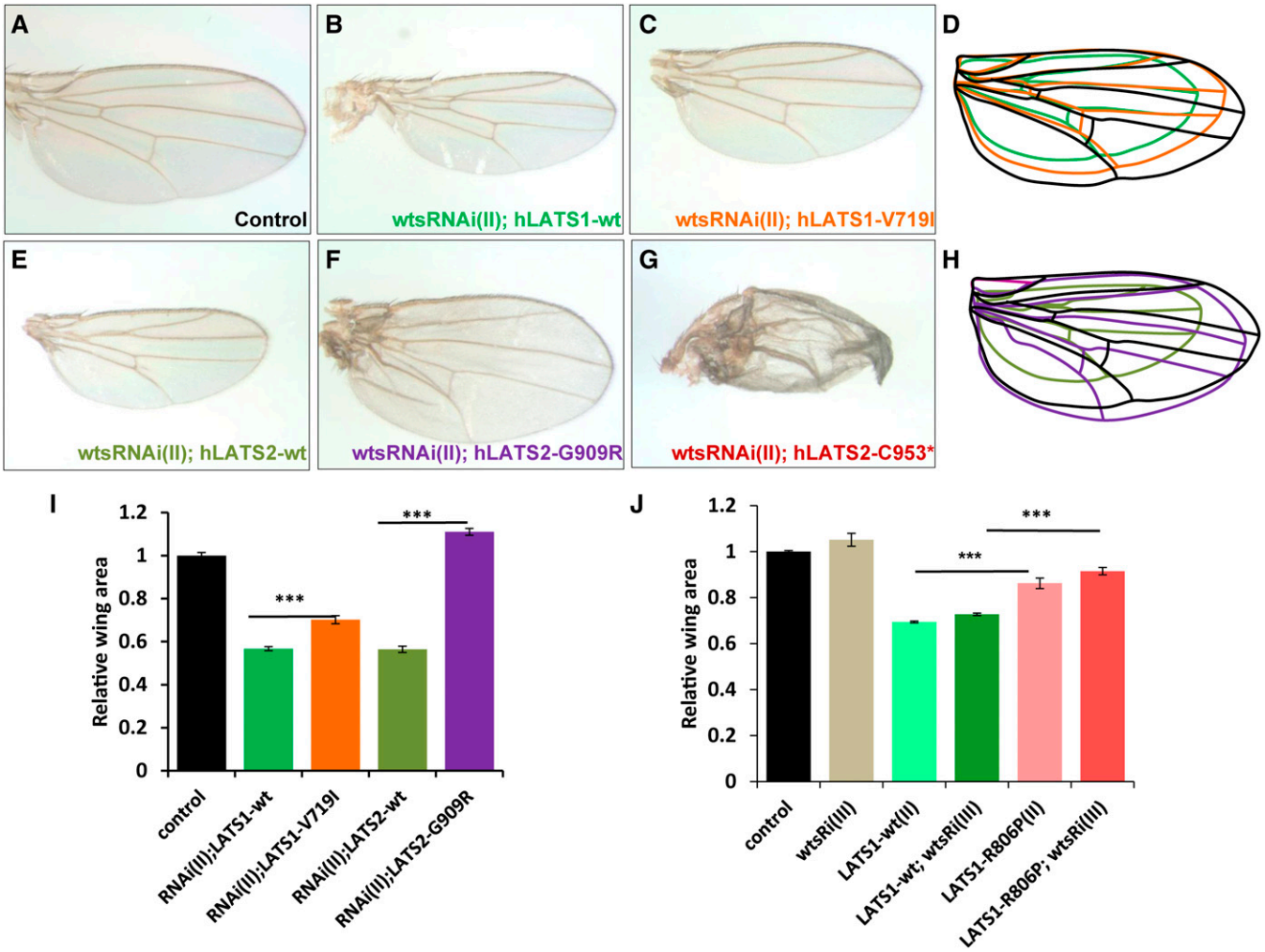

Figure 3 *In vivo* analysis of hLATS1/2 and variants for their activity to control organ size in the absence of the endogenous *wts* gene function. Representative images of female adult wings (anterior to the top). (A) *MS1096-Gal4/+*; *UAS-lacZ/+*, which only expresses a *UAS-lacZ* transgene in the wing. β-Galactosidase expression in the wing has no impact on wing development. (B) *MS1096-Gal4/+*; *UAS-wtsRNAi(II)/+*; *UAS-hLATS1-wt*. (C) *MS1096-Gal4/+*; *UAS-wtsRNAi(II)/+*; *UAS-hLATS1-V719I*. (E) *MS1096-Gal4/+*; *UAS-wtsRNAi(II)/+*; *UAS-hLATS2-wt*. (F) *MS1096-Gal4/+*; *UAS-wtsRNAi(II)/+*; *UAS-hLATS2-G909R*. (G) *MS1096-Gal4/+*; *UAS-wtsRNAi(II)/+*; *UAS-hLATS2-C953**. Wing size comparison of samples from A–C is shown in D, and those from A, E, and F are shown in H. (I and J) Display wing size data from statistical analysis of flies with genotypes described at the bottom portion of the panels ($n = 40$ for each genotype). (J) *UAS-wtsRNAi* (III, inserted on the third chromosome) driven by *MS1096-Gal4* did not cause an obvious lethality and therefore *UAS-hLATS1-R806P* (II, inserted on the second chromosome) was not tested in a survival rate experiment. Activities of LATS1-R806P in overexpresion and wing-size rescue experiments were analyzed and are shown in J. Results represent mean ± SEM. $^{**}P < 10^{-4}$, $^{***}P < 10^{-22}$.

due to a dominant-negative effect. LATS2-C953* failed to maintain a normal wing morphology in *wts* mutant tissues (Figure 3G), which makes it difficult to accurately measure its wing size. These results are consistent with what was observed in other assays described in Figure 1 and Figure 2.

Mammalian Lats1/2 genes mediate growth control by directly phosphorylating and inactivating Yap/Taz (Dong *et al.* 2007; Zhao *et al.* 2007; Hao *et al.* 2008; Lei *et al.* 2008). To investigate whether the mutations in *hLATS1/2* affect their ability to negatively regulate YAP, we have used luciferase reporter assays to directly measure YAP/Yki activities. In cultured HEK293T cells, wild-type hLATS1 and hLATS2 effectively inhibited the transcriptional activity of YAP (Figure 4A). V719I and R806P mutations in hLATS1 and G909R and C953* in hLATS2 all decreased the activity of hLATS1/2 as inhibitors of YAP (Figure 4A). As expected, YAP activity was not obviously affected in cells expressing hLATS2-G40E (Figure 4A). Similar results were observed using *Drosophila* S2 cells (Figure 4B). Therefore, these data further support that the *hLATS1/2* mutations cause a loss or reduction of their activity in mediating Hippo signaling.

Genetic analysis of Lats/Wts family genes using *Drosophila* and mice models has revealed their role as a negative growth regulator and tumor suppressor (Justice *et al.* 1995; Xu *et al.* 1995; St John *et al.* 1999). The fact that hLATS1 can functionally replace Wts in *Drosophila* strongly suggests that LATS may function as a tumor suppressor in human (Tao *et al.* 1999). Although they are not frequently mutated

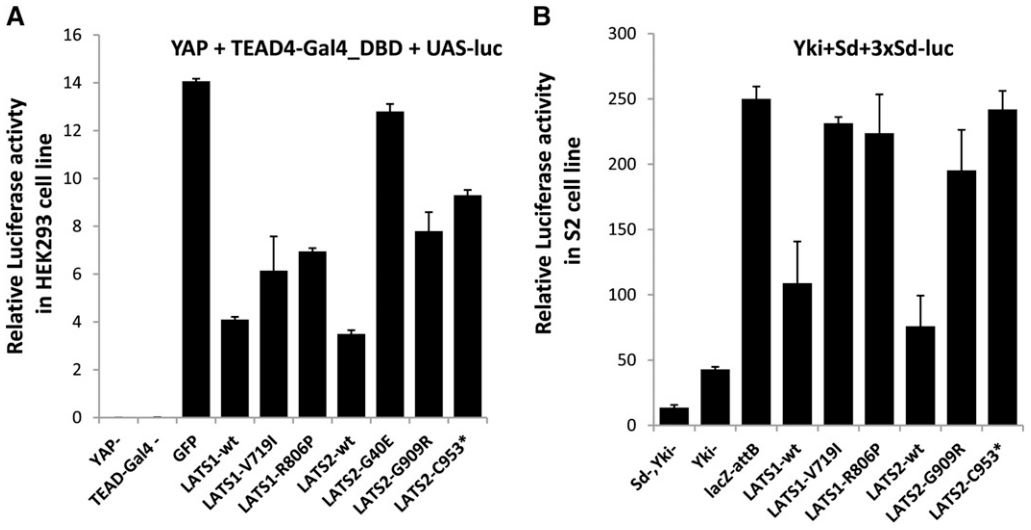

Figure 4 Activities of hLATS1/2 and variants in regulating YAP/Yki transcriptional coactivator activity in both human and *Drosophila* cells. (A) Relative luciferase activity in HEK293T cells that overexpressed Renilla luciferase as an internal control, *UAS-luciferase*, *CMV-YAP* (except the first column), and fusion construct *TEAD4-Gal4* that fuses *TEAD4* with *Gal4* DNA-binding domain (except the second column). (B) Relative luciferase activity of *UAS-LATS1/2*-wild type and mutants in S2 cells that overexpressed Renilla luciferase as an internal control, *3xSd (Scalloped)-luciferase*, *Ac-Yki* (except the first and second columns), and *UAS-Sd* (except the first column). HEK293T cells were maintained in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum and 0.5% penicillin–streptomycin. They were transiently transfected using PolyFect Transfection Reagent (Qiagen) and harvested 36 hr later. Plasmids include wild-type or mutant *hLATS1* and *hLATS2* in *pcDNA3.1-Hygro-3xFLAG*, *pCMV-YAP*, *pCMV-Gal4-TEAD4*, *pUAS-LUC*, and *pCMV-Renilla* (gift from Kun-Liang Guan). Cells were lysed after 36 hr and luciferase activity was detected using the Dual-Luciferase Reporter assay kit and GloMax multi detection system (Promega). *Drosophila* S2R$^+$ cells were cultured in Schneider's medium (Sigma), containing 10% fetal calf serum. S2 cells were transfected using Effectene (Qiagen) with wild-type or mutant *hLATS1/2* in *pUAST-attB*, *pAc-Yki*, *pUAST-Sd*, *p3xSd-LUC*, and *copia-Renilla* (gift from Jin Jiang), together with *pAc-Gal4*. Results represent the mean of standard error of three independent experiments.

genes, an increasing number of mutations in *LATS1* and *LATS2* have been detected in human cancers through human cancer genome projects. In this work, we have shown that *LATS1* and *LATS2* mutations from human cancer can lead to loss or reduction of their growth-inhibitory activity. Therefore, human *LATS1* and *LATS2* mutations are expected to confer tissue growth advantage to drive tumor development.

Acknowledgments

We acknowledge Johannes Bischof for fly lines; Kun-Liang Guan, Jin Jiang, and Xiaolong Yang for plasmids; Nicholas Anzalone for figure editing; and the Bloomington *Drosophila* Stock Center and Vienna *Drosophila* RNAi Center. This work was partly supported by the National Science Foundation.

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Communicating editor: I. Hariharan

GENETICS

Supporting Information
http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156372/-/DC1

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DOI: 10.1534/genetics.113.156372

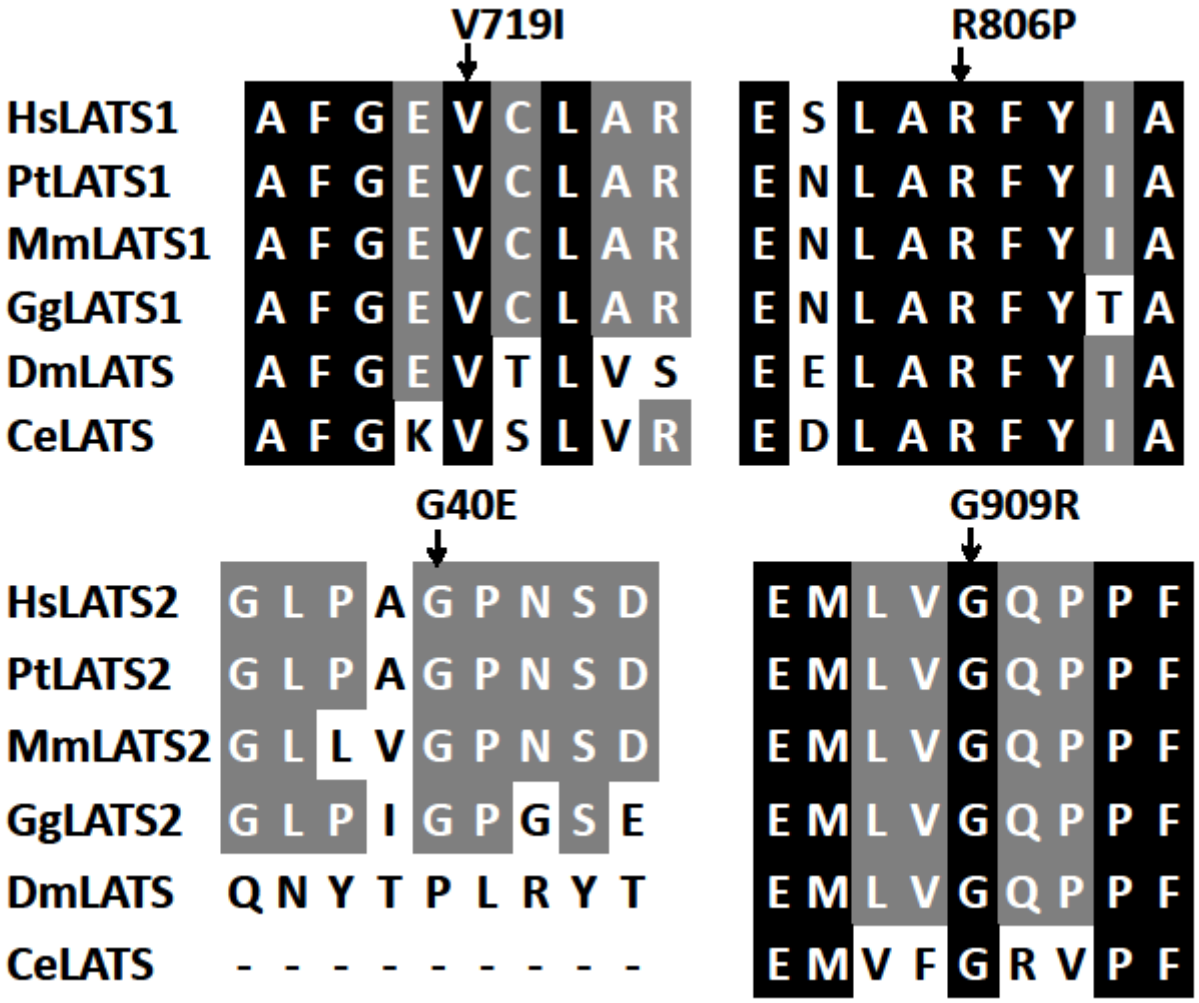

Figure S1 Evolutionary conservation of amino acids that are mutated in human LATS1 and LATS2. Human LATS1 (top) and LATS2 (bottom) protein sequences that cover several mutated amino acids are aligned and compared with homologous sequences from other vertebrates, Drosophila and nematode. For vertebrate Lats1, they include *H. sapiens* NP_004681.1, *P. troglodytes* XP_001173355.1, *M. musculus* NP_034820.1 and *G. gallus* XP_419666.2. Vertebrate Lats2 includes *H. sapiens* NP_055387.2, *P. troglodytes* XP_001149147.1, *M. musculus* NP_056586.2 and *G. gallus* XP_417143.3. For *D. melanogaster* Lats/Warts: NP_733403.1. For *C. elegans* Lats/Wts: NP_492699.1. Protein sequence alignment was performed by using MEGA 5.2.1 (TAMURA *et al*. 2011).

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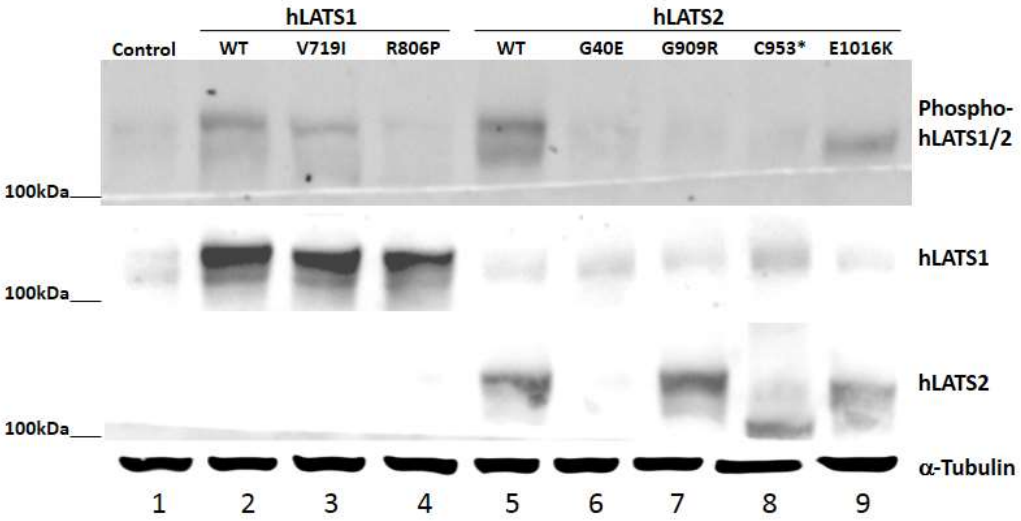

Figure S2 Western blot analysis of human LATS1 and LATS2 proteins in HEK293T cells. HEK293T cells were transfected with wild-type (lane 2 and lane 5) or mutant (lane 3-4 and lane 6-9) *hLATS1/2* DNA constructs as indicated. Anti-hLATS1 and anti-hLATS2 antibodies were used to specifically detect hLATS1 and hLATS2 expression, respectively (the second and third panels). The endogenous hLATS1but not hLATS2 protein was expressed at a detectable level. An anti-Phospho-LATS1/2 (Thr1079/1041) specifically identified activated hLATS1 and hLATS2 kinase proteins (the top panel). As all hLATS1/2 constructs wee tagged with a Flag sequence, their expressions were all verified by an anti-FLAG antibody (data not shown). Expression of a-Tubulin was monitored to ensure equal loading of protein extracts (the bottom panel). Untransfected HEK293T cells were used as a negative control (lane 1). Protein markers are shown on the left side of the panels.

pcDNA3.1-hygro-3xFLAG-hLATS1 and *pcDNA3.1-hygro-3xFLAG-hLATS2* (gift from Dr. Xiaolong Yang) were used for site-directed mutagenesis to generate V719I and R806P mutant constructs for *hLATS1* and G40E, G909R, C953* and E1016K mutants for *hLATS2*. *hLATS1/2* wild-type and mutant variants were cloned into *pUAST-attB* and verified by DNA sequencing.

For Western blot analysis HEK293T cells were transfected using PolyFect (Qiagen). After 36 hours, cells were treated with 1.0 μM okadaic acid (OA) (Sigma) for 30 min at 37°C, and harvested 36 hours later in lysis buffer (150 mM NaCl, 50 mM Tris-HCl [pH = 7.4], 2 mM EDTA [pH = 8.0], 1% Triton-X 100, 10% glycerol, 2 mM DTT, 1 mM PMSF, 10 mM NaF, 2 mM Na_3VO_4, 60 mM Glycerol-2-phosphate, containing Protease inhibitors and phosphatase inhibitor (Sigma). The following antibodies were used: anti-LATS1 (Santa Cruz), anti-LATS2 and anti-Phospho-LATS1/2 (Thr1079/1041) (Cell Signaling), anti-FLAG and anti-a-Tubulin (Sigma).

Table S1 Detail information on non-synonymous mutations for human *LATS1* (58 samples) and *LATS2* (43 samples) genes collected in the COSMIC database (http://cancer.sanger.ac.uk/cancergenome/projects/cosmic/) is presented. Mutations that have been experimentally analyzed in this study are highlighted with bold (hLATS1: V719I and R806P; hLATS2: G40E, G909R and C953*).

Table S1 is available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156372/-/DC1

Column A: Names of cancer samples.

Column B: Mutations of specific amino acids in hLATS1 or hLATS2 open reading frame. "*" represents a stop codon. "fs*" represents a frame shift followed by certain number of amino acids before a newly introduced stop codon.

Column C: specific nucleotides that have been substituted or deleted.

Column D: Primary tissues where cancers were identified.

Column E: Tissue subtypes.

Column F: Cancer types

Column G: Cancer subtypes

Column H: PubMed identification numbers for relevant references.

Column I: Somatic status - information on a mutation being somatically induced or not.

Column J: Sample source - a sample being derived from tumor tissue or cell line.

Column K: Zygosity - information on whether a mutation is homozygous or heterozygous within the sample.

Table S2 ***in silico*** **analysis of** ***hLATS1*** **and** ***hLATS2*** **mutations from the COSMIC database.** Mutations that have been experimentally analyzed in this study are highlighted with bold (hLATS1: V719I and R806P; hLATS2: G40E, G909R and C953*). While being predicted to be less significant changes, the other ones have been only computationally analyzed. We initially focused on these *hLATS* mutations as they were the only ones available at an early stage of this study.

Table S2 is available for download at http://www.genetics.org/lookup/suppl/doi:10.1534/genetics.113.156372/-/DC1

Column A: Gene names - human *LATS1* and *LATS2* genes.

Column B: Cancer sample names.

Column C: Mutations of specific amino acids (AA) in hLATS1 or hLATS2 open reading frames. "*" represents a stop codon.

Column D: LATS protein domains or regions where mutations are located.

Column E: information on site conservation in the *Drosophila warts* gene.

Column F: Counts of the methods shown in Columns G-J, which predicts a mutation to be disrupting or damaging to protein function, based on conservation and structure information.

Column G: The SIFT program (http://sift.jcvi.org/www/SIFT_enst_submit.html) [1].

Column H: The PolyPhen-2 program (http://genetics.bwh.harvard.edu/pph2/) [2].

Column I: The Mutation Assessor program (http://mutationassessor.org/?set=tcga-gbm-nov.2009) [3].

Column J: The SNAP program (https://rostlab.org/services/snap/) [4].

Columns K-M: Information in database on SNP's found in the same amino acid position.

Column N: Reference information.

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