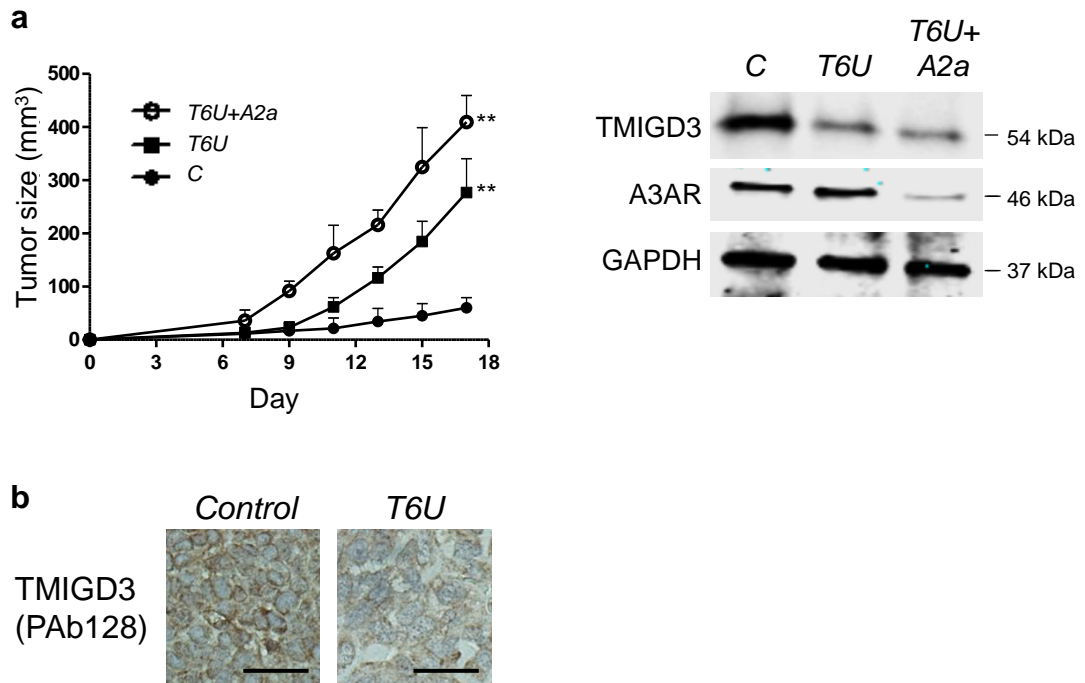
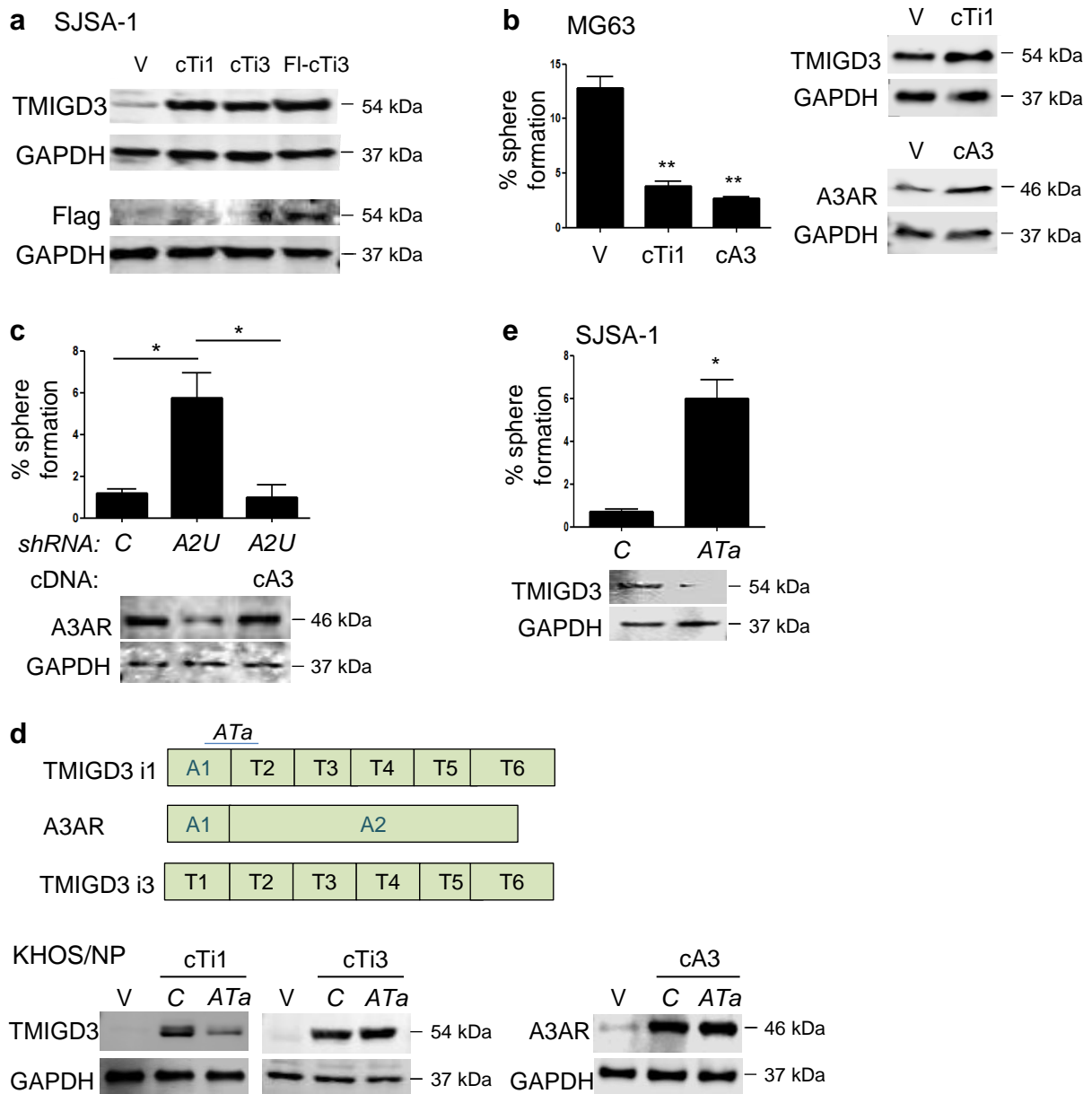


Supplementary Figure 1 | Knockdown of TMIGD3 or A3AR increases sphere formation in multiple OS cells.
(a) TMIGD3 i1 protein and location of the TMIGD3-specific antibody (PAb128, red bar). **(b)** Sphere formation assays using MG63 cells infected with lentiviral vectors encoding non-silencing *control* (C) or *TMIGD3* (T6U) shRNAs (left). Error bars: means \pm S.D. (n=3 independent experiments). ** p<0.01; Student's t-test (two-tailed). Representative images of western blotting for TMIGD3 and GAPDH (right). **(c, d)** Sphere formation assays using Saos2 **(c)** and MG63 **(d)** cells downregulated for A3AR (A2a, A2b). Graphs show percentage of sphere formation. Representative images of western blotting for A3AR and GAPDH are present below the graph. Error bars: means \pm S.D. (n=3 independent experiments). * p<0.05, ** p<0.01; Student's t-test (two-tailed).



Supplementary Figure 2 | Downregulation of TMIGD3 and A3AR is associated with OS malignancy *in vivo* and clinically.

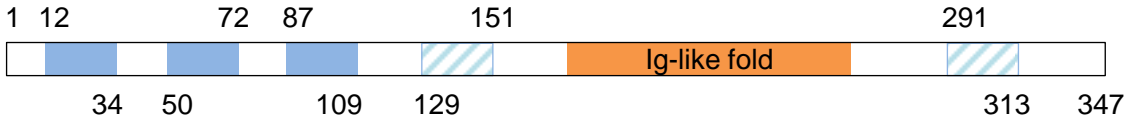
(a) Tumor growth of SJSA-1 cells downregulated for TMIGD3 alone (*T6U*), or in combination with A3AR (*T6U+A2a*). Cells (1×10^6) were subcutaneously injected into nude mice, and sizes of tumors were measured twice a week for 17 days. Error bars: means \pm S.D. ($n=5$ mice per group). ** $p < 0.01$; Two-way ANOVA. Representative images of western blotting for TMIGD3, A3AR, and GAPDH (right). (b) IHC for TMIGD3 with PAb128 using SJSA-1-derived tumors infected with lentiviral vectors encoding non-silencing (*control*) or *TMIGD3* (*T6U*) shRNAs. Scale bar: 100 μ m.



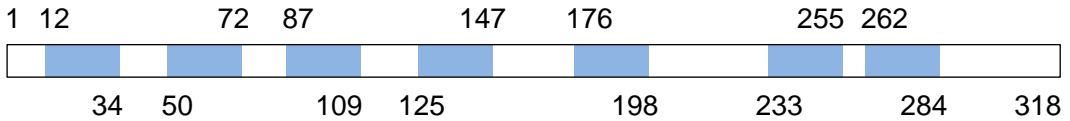
Supplementary Figure 3 | Manipulation of TMIGD3 and A3AR expression and its effects on sphere formation.

(a) TMIGD3 i1 and i3 run at a similar size. Representative images of western blotting using SJSa-1 cells expressing vector control (V), TMIGD3 i1 (cTi1), TMIGD3 i3 (cTi3), or Flag-tagged cTi3 (FI-cTi3) for indicated proteins. (b, c) Sphere formation assays using MG63 cells overexpressing vector control (V), TMIGD3 i1 (cTi1), or A3AR (cA3, b) and SJSa-1 cells following knockdown of A3AR with a shRNA targeting the 3' UTR (A2U) and concomitant overexpression of A3AR (A2U + cA3) (c). Graph showing % of sphere formation. Representative images of western blotting for TMIGD3, A3AR, and GAPDH. Error bars: means \pm S.D. (n=3 independent experiments). * p<0.05, ** p<0.01; Student's t-test (two-tailed). (d) Design of the ATa siRNA that targets only TMIGD3 i1 with minimal effects on TMIGD3 i3 and A3AR. Immunoblots for TMIGD3 and A3AR using KHOS/NP cells overexpressing TMIGD3 i1 (cTi1), TMIGD3 i3 (cTi3), or A3AR (cA3) following transfection of control or ATa siRNAs. (e) Sphere formation assays using SJSa-1 cells following transfection of the ATa siRNA. Representative immunoblots showing knockdown of TMIGD3 (below). Error bars: means \pm S.D. (n=3 independent experiments). * p<0.05; Student's t-test (two-tailed).

TMIGD3 i1

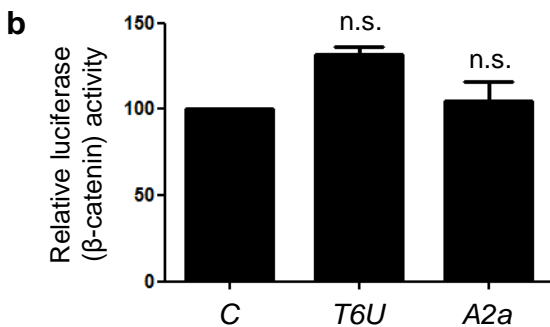
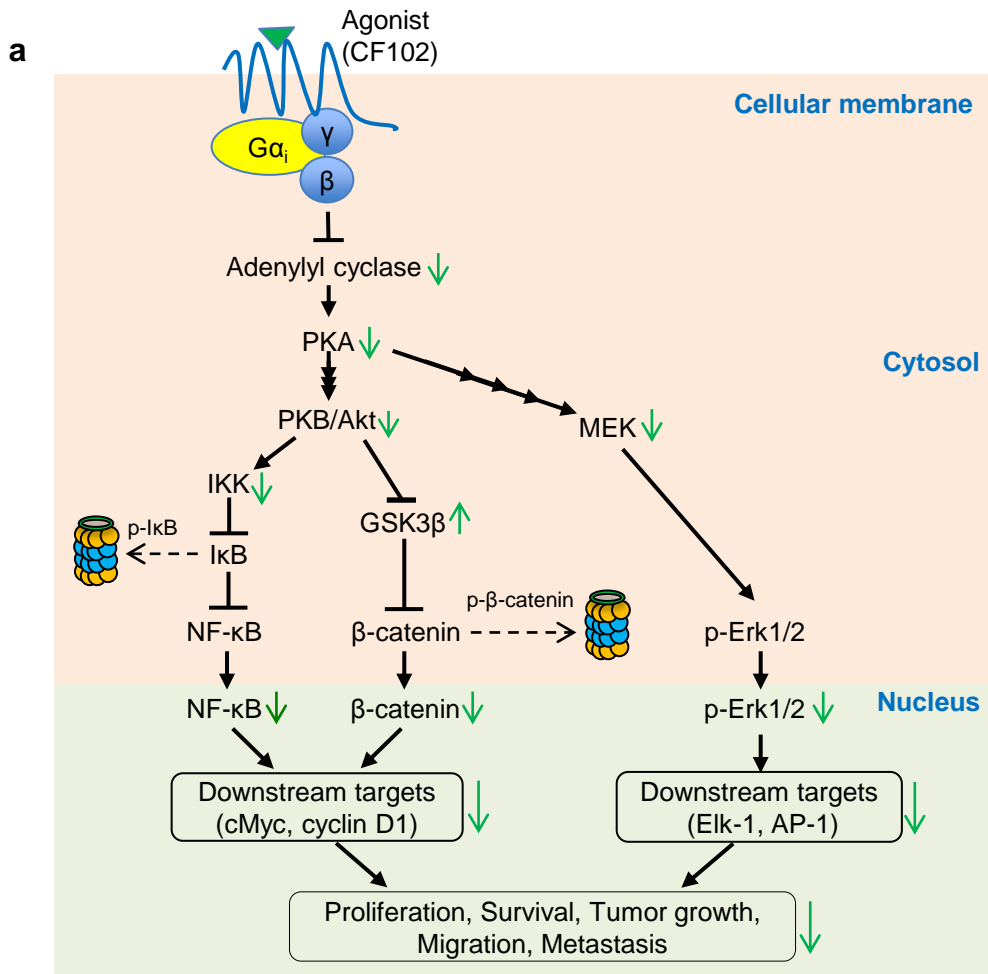


A3AR



Supplementary Figure 4 | Predicted structure of TMIGD3 i1 and its comparison with A3AR.

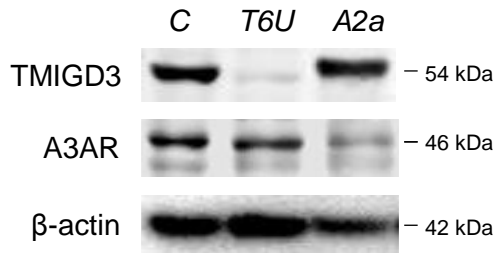
Blue filled boxes indicate reported transmembrane helices. Blue boxes with oblique lines indicate predicted transmembrane helices. Three N-terminal transmembrane helices are common between TMIGD3 i1 and A3AR. Orange box indicates the Ig-like fold. Numbers indicate amino acid locations.



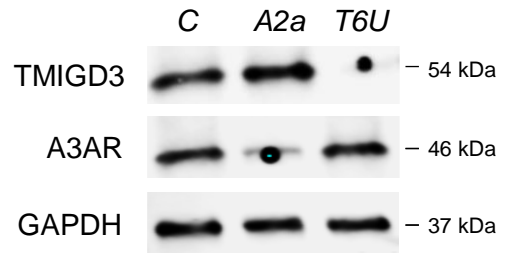
Supplementary Figure 5 | Signaling pathways regulated by A3AR and effects of knockdown of TMIGD3 or A3AR on the β -catenin activity.

(a) Cancer associated-signaling pathways regulated by A3AR. (b) TOPFlash luciferase reporter assays for measuring the β -catenin activity in SJSA-1 cells downregulated for TMIGD3 (*T6U*) or A3AR (*A2a*). Graph showing relative luciferase activity normalized to that of SJSA-1 cells infected with non-silencing *control* (*C*) lentiviral vector. Error bars: means \pm S.D. ($n=3$ independent experiments). n.s.: not significant; Student's t-test (two-tailed).

a SJSA-1

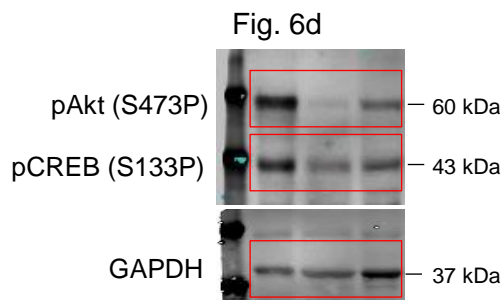
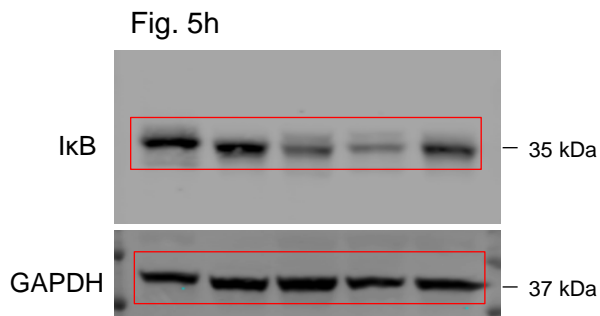
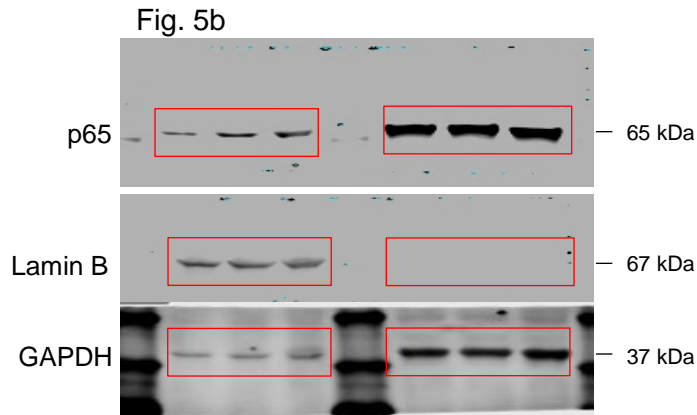
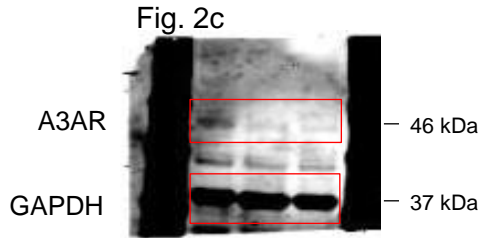
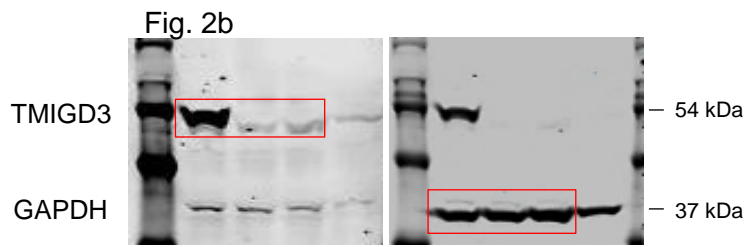


b Saos2



Supplementary Figure 6 | Knockdown of TMIGD3 (*T6U*) or A3AR (*A2a*) does not alter protein levels of A3AR or TMIGD3, respectively.

(a, b) Representative images of western blotting for TMIGD3, A3AR, β -actin, and GAPDH in SJSA-1 (a) and Saos2 (b) cells downregulated for TMIGD3 (*T6U*) or A3AR (*A2a*).



Supplementary Figure 7 | Uncropped images of important western blotting presented in the main figures.

Red boxes indicate the areas presented in the representative figures.

Supplementary Table 1 | Sphere forming potential of OS cell lines

| Cell line | Total cell # examined | % sphere formation* (average +/- S.D.) 30-75 μm, >75 μm |
|------------------|------------------------------|---|
| U2OS | 140,000 | 0+/-0, 0+/-0 |
| Saos2 | 140,000 | 0+/-0, 0+/-0 |
| SJSA-1 | 140,000 | 0.5+/-0.5, 0+/-0 |
| MG63 | 2,160 | 7.7+/-1.5, 4.5+/-2.1 |
| KHOS/NP | 1,780 | 9.2+/-2.1, 7.7+/-1.8 |

Spheres < 30 μm in diameter were not counted as spheres.

* % sphere formation: a percentage of # of spheres formed/# of cells seeded.

Supplementary Table 2 | Functions of candidates

| Gene name (abbreviation) | Function |
|---|---|
| Transmembrane and immunoglobulin domain containing 3 (TMIGD3) | Unknown. Ig-like fold containing protein family. TMIGD3 i1 is an alternative splicing form of adenosine A3 receptor (A3AR), whereas TMIGD3 i3 has no overlap with A3AR. |
| Solute carrier family 45-4 (SLC45A4) | Unknown. No reference. |
| Spi-B (Spi-B) | Ets family transcription factor implicated in B cell lymphoma suppression. |
| Glutamate receptor interacting protein 1 (GRIP1) | Cytoplasmic multi-PDZ scaffolding protein implicated in neuronal synaptic function. |
| Zinc finger RNA binding protein 2 (ZFR2) | Unknown. No reference. |
| Spermatogenesis and centriole associated 1 (SPATC1) | Centrosomal protein implicated in zygotic cell division (Y chromosome). |
| Periphilin-1 (PPHLN1) | Gastric cancer antigen Ga50 involved in epithelial differentiation. |