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Peer Review File

Epstein-Barr virus infection upregulates extracellular OLFM4 to activate YAP signaling during gastric cancer progression

Corresponding Author: Professor Zhaocai Zhou

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, authors identified olfactomedin 4 (OLFM4) containing large extracellular vesicles (EVs) induced by Epstin-Barr virus (EBV) infection in gastric cancers (GCs). Authors revealed that OLFM4 as a direct target gene of the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway. Authors further demonstrated that MV-containing OLFM4 as an extracellular inhibitory ligand of Hippo pathway.

The study is interesting, and the manuscript is well-written. There are some major and minor points to be addressed in order to improve the quality of study.

1) Figure 3c, d, quality of STING immunoblot needs to be improved.
2) Figure 3f, OLFM4 immunoblot in response to RU.521 treatment was not clear.
3) Figure 4, because the EVs usually are circulated in the blood, can OLFM4 be detected in the EBV positive GC patients' serum or blood samples?
4) Figure 5, align with above comment, the effects of OLFM4 containing EVs on tumor formation needs to be detected by intravenous injection.
5) Line 248, Authors have described:"5x106 HGC-27 cells were injected into nude mice.."
Line 596, Authors have described:"HGC-27 cells (1x106 cells in 100 μl medium) were injected into flanks of 4-week-old male Balb/c nude mice.." the cell numbers in animal works need to be clarified.
6) According to Hippo-YAP regulation theory, YAP predominantly localizes in the nuclear under the low cell density condition. However, authors showed YAP extensively excluded from nuclear even in the sparse cell growth condition (Figure 6i, top). Can author explain why this happened? Was intrinsic Hippo signaling impaired in this cell line?

Reviewer #2

(Remarks to the Author)
In this study, Wen et al. found that EBV infection activates cGAS-STING signaling to induce the expression of OLFM4, which can be secreted outside of the cells via MVs. OLFM4-carried MVs act on the cancer cells to inhibit the Hippo-Yap pathways, promoting cell proliferation and tumorigenesis. OLFM4 binds to FAT1 to abrogate its intracellular interaction with MST1, leading to enhanced YAP activation. The following concerns need to be addressed to improve the rigor of the study.
Major concerns:
1. What cell types are infected by EBV in the TME? While the histology data showed that OLFM4 is highly expressed in EBV++ cancers, the cell types that are infected by EBV are unclear. Based on the cell culture experiments, it seems that both cancer cells and epithelial cells can be infected by EBV. Then, what is the source of the cells that produce OLFM4? If cancer cells produce OLFM4 on their own, what are the roles of intracellular OLFM4 in cancer cell proliferation? Can intracellular OLFM4 inhibit Hippo-YAP signaling?
2. Herpesvirus establishes a widespread infection in adult individuals. It is likely that EBV+ patients may also be infected by other herpesviruses. Can other herpesviruses induce OLFM4 expression? How can the authors rule out the impact of other herpesvirus infections on OLFM4 induction?
3. In Figure 3c-d, the molecular weight of STING seems incorrect. The STING oligomers and IRF3 dimers is unclear, and

more convincing images should be provided.
4. In Figure 3e, the OLFM4 expression only marginally decreased in STING knockdown cells. This might be caused by residual STING expression. To confirm that EBV-mediated OLFM4 induction is indeed STING-dependent, the authors should generate STING knockout cells and confirm OLFM4 expression in WT vs. STING KO cells in response to EBV infection.
Minor concerns:
1. In Figure 2A, could the author explain why GAPDH is present in the supernatant?
2. Statistical analysis is required for Figure 5g.

Reviewer #3

(Remarks to the Author)
In the manuscript by Wen er al. the authors describe how EBV in gastric cancer cells results in Olfactomedin 4 (OLFM4) positive microvesicle (MV) production. OLFM4 is activated by EBV through cGAS-STING signalling and this, in turn, promotes gastric cancer progression. Finally, the authors provide some data that OLFM4 inhibits HIPPO signal transduction. Overall, this is a multi-component cell biological study with some correlative clinical data. While interesting, I am a bit lost whether the MV component is connected to some other aspects of the study. That is to say, the authors mingle tissue positivity for OLFM4 and infer roles it might have (or the mere presence) in MVs. This disconnect is very obvious. Additionally, there is a large amount of important information and data withheld which does not increase the trust in the manuscript.

A few key points:
Methods: the method section is comprehensive, however, lacks all details to the specifics of the reagents used. For example, lot, catalogue, or version information is completely absent and needs to be added.

All figures/legends are missing the information on the number of technical and biological repeats completely. Please refrain from non-informative lines like ‚all lanes were loaded with the same amount of protein' but provide the specifics that whatever amount was used. For MV specific analysis, why is same protein and not Same particle number used as reference? It is strongly suggested that in MV data, the same particle amount should be used, or at least referenced, as in ‚so much protein is loaded in all lanes which equal x, y and z particles in the lanes, respectively.'

Would the authors please provide all western images in the extended data in an uncropped version. Also additional high mag of TEM images (to show more than 1) and/or lower mag images should be provided to demonstrate the general appearance of the MVs

Figure1
1e: The CD63 blot: how was this western done? Most CD63 antibodies require non-denaturing conditions, which are not described in the methods and the bands for CD63 do not look as these most commonly shown by authors for MV and other EV data.
1g requires labels to indicate the protein identities shown.
1f/g also require a method-independent validation of the MV data.
1f indicates that MMP7 is even more promising candidate than OLFM4 on MVs and if the work focuses on the MVs as secretion pathway and mediator of the HIPPO pathway functions as per later, this should be assessed.
1i: Why is a proteomic assessment of MV content shortlisted by gene expression changes in cancer samples? I cannot understand the reasoning as several EV publications show that the correlation between cell/tissue and EV/MV abundance of proteins (and RNA) cannot be assumed-which exactly is done here. . The argument that the MMP7 levels are lower in cells (1J) is therefore confusing: as mentioned above, is the investigation on the role of OLFM4 in MVs or cells or both? No data is provided to demonstrate any correlations between cell and MV levels for OLFM4, MMP9 or any of the other shortlisted hits. Could it be that MMP7 or other MV hits having a log-scale higher fold change could be at more abundance, and therefore at least as important, to MV functions in EBV GC than OLFM4?

A general question to the data in Fig1 and elsewhere: EV isolation protocols, both for small and large EVs, could, in principle, co-isolate virus particle. Have the authors evaluated if EBV GC cells or tissues either secrete EBV (and it is a co-isolate/contaminant) or whether it is contained within MVs? Are the recipient cells in later experiments EBNA positive after MV administration of EBV GC cells?

Fig2a/b: This experiment should a) be shown for more than 1 example and b) for EBV negative GC tissues. Some data is okay to be added to extended data. In 2a, could the authors please show the same MV markers/cell markers as in Fig1e

In Fig2, the abundance of MMP7 and other proteins with higher abundance in MVs than OLFM4 should be shown.
Fig2/text: An explanation for the non-GC specialist reader about the characteristics of HGC-27, GES-1 and other lines should be included.

Fig3:
Can EBNA in recipient cells or EBV in MV isolates be detected?

Fig4: I still cannot understand the constructed relationship between OLFM4 on MVs and for example this tissue/patient data. Also, all MVs were either tissue or cell line derived. Do the authors suggest that the MV function, and therefore the OLFM4-

MVs, only act in the local, immediate environment of the producing cell? Can OLFM4-MVs be found in the draining lymp nodes or the blood? For this figure, what are the OLFM4 levels on MVs in the tissues?

Fig5/extended data 2: Why are HEK293T cells used to overexpress OLFM4? What is the use? The explanation is absent and I am left wondering if this data was just ‚there' and put in to use it, or if there is any meaning to it. The only conclusion is that the 293 derived MVs are used in Fig5a, c, e, f, g. If this is the case, this is even more concerning as now the conclusion is that OLFM4 regardless of reason might have an effect exclusively (?) on GC cells. Are the MVs associating with the HGC27 cells, are they taken up, how many MVs are used/cell, how often repeated.
5b, d: What was done here? WT HGC cells (WITH OLFM4 MVs) were treated with MVs derived from knockdown cells. First of all, there is no data anywhere showing the efficacy of the cellular knockdown, neither the effect of MV levels. Then, the use of a reduced OLFM4-MV (assuming this might be the case) is impacting on cells producing OLFM4 positive (high) MVs? How? Are the OLFM4 reduced MVs preferentially taken up? This whole figure, in the current form, raises many more questions about the rigor of the data acquisition than it adds to the scientific knowledge gain.

Based on the above, is it correct to assume that the OLFM4 overexpression derived MVs are also 293 cells are used? First of all, there is no mentioning, apart from the first confusing lines in the results text for Fig5 that 293 cells are used. Why is this omitted? Then, the authors use 293T cells-is the T-antigen included in MVs? Have evaluations been done between the different 293 derived (with or w/o OLFM overexpresison) MV on a proteomic level? Would the mismatch between 293T MVs and hgc27 cells result in immune activation, which through the expression of OLFM4 might be altered? Especially as the authors investigate interferon responses, this is all very challenging to interpret.

Discussion: The authors might want to explore, in more detail, the role MVs or other EVs could play in cancer progression and stromal responses. Re-statement of results should be the exception and it is suggested to the authors to re-work the current discussion to focus more on the implications of the data and similar/opposing results from others rather than explaining their own data again.

Minor point: The sentence line 155-157 does not make sense

Reviewer #4

(Remarks to the Author)
In this study, Wen et al. proteome profiling of microvesicles (MVs) in Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC). They found that OLFM4 is a critical protein induced by EBV infection and secreted via MVs, and promotes gastric tumorigenesis through
Hippo-YAP signaling. OLFM4 was a downstream target gene of cGAS-STING pathway that was activated upon EBV infection, and MV-carried OLFM4 was an extracellular inhibitory ligand for the Hippo pathway. They found that viral infection coupled with extracellular vesicles plays a role in control of Hippo signaling, highlighting OLFM4-Hippo axis as a potential therapeutic target for EBV associated cancers.

General comments:
Contribution of MV-carried protein in controlling Hippo signaling and tumorigenesis is potentially interesting especially in the field of virus associated tumor, although essence of this manuscript including importance of extracellular vesicles, involvement of cGAS-STING pathway in viral infection, contribution of OLFM4 in gastrointestinal cancer, does not seem so novel and may not give strong impact. The comments below are the concerns to be addressed.

Specific comments:
1. EBV is infected to HGC-27, and RNA-seq was conducted (in Fig. 3). Although infection rate of EBV in gastric cells is generally so low, but did the authors select infected cells by drugs? Materials and methods showed that they used Akata cells for infection experiments, but it is not clear whether EBV in Akata cells is recombinant EBV or not. RNA was extracted at 24 h after infection (Fig. 3a,b), but it is not clear whether this is the effect of EBV “infection” or exposure to foreign substances. Similarly, elevation of OLFM4 was shown (Fig 3d) under extraction of proteins at 48 h, but it is not clear whether this is the effect of EBV “infection” or exposure to foreign substances. Infection rate might be shown, e.g. by EBER in situ hybridization, copy number of EBV genome in cells might be shown, e.g. by qPCR, and EBV-infected cells can be selected, by utilizing drug-resistance.
2. MV treatment on GC cell line was performed using HGC-27 (Fig. 5a) without replicate. This experiment should be done using multiple cell lines, by adding at lease another cell line.
3. shRNA knockdown on MV-treated GC cell line was performed (Fig. 5b), but this was also conducted using HGC-27 only. The authors should do this experiment using multiple cell lines, by adding at lease another cell line.
4. shRNA experiment is also very confusing and unclear. Fig. 5b legend shows “30 μg HGC-27 shCtrl or shOLFM4 cell lines derived MVs were used for the treatment”, but were shCtrl and shOLFM4 treated with HGC-27? If so, why MV-treated HGC-27 must be treated by shOLFM4? Or, was shRNA treated with HEK293T? If so, the target of shOLFM4 would seem to be overexpressed OLFM4 in HEK293T cells, but such experiment is usually done overexpressing function-lost OLFM4, not shRNA knockdown.
5. Perturbation experiment on OLFM4, such as shOLFM4, should additionally done on EBV-positive GC cell lines, and EBV-infection model.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Authors have successfully addressed the reviewer's comments. The manuscript is ready to be accepted for publication.

Reviewer #2

(Remarks to the Author)
The revised manuscript has been much improved and addresses some of my concerns. The authors propose a model in which cancer produces OLFM4-carried MVs that bind to the extracellular domain of FAT1 on recipient cells to inhibit Hippo signaling. However, it remains unclear whether OLFM4 is embedded inside the vesicle or anchored on its surface. OLFM4 can only exert its functional activity if it is localized on the vesicle membrane surface. Alternatively, OLFM4-carried MVs might be engulfed by the recipient cell, and released intracellular OLFM4 can also inhibit Hippo signaling, as demonstrated by the authors' new data. How does intracellular OLFM4 inhibit Hippo signaling? It appears to do so through mechanisms distinct from those of extracellular OLFM4. The authors should provide clear evidence demonstrating whether the inhibition of Hippo signaling in the recipient cells is caused by extracellular or intracellular OLFM4-carried MVs.

Another minor question is why the cGAS-STING pathway is suppressed by HSV1 infection. It is widely known that HSV1 can activate cGAS-STING signaling.

Reviewer #3

(Remarks to the Author)
The authors of the manuscript "Olfactomedin 4 is a direct target of the cGAS-STING pathway and acts as an extracellular inhibitor for the Hippo signaling in EBV-associated gastric cancer" have made a tremendous effort addressing the various comments and concerns of the reviewers, including mine.
While most of the points raised in my previous assessment are addressed, I want to reiterate that the use of 293T derived EVs is a concern. Referring to a couple of publications by others as statement that this was used previously and therefore is 'fine' is questionable. However, instead of instigating a complete immunological evaluation, which would be utterly out-of-line for the scope of the manuscript and the already very comprehensive complexity, would the authors be able to amend the (much improved) discussion to touch on the limitations and implications of using EVs (even low speed MVs) from virus infected cells (referring to my concerns about EBNA/EBV) and 293T donor cells? It is certainly something we, as a field, need to keep in mind, and if this important gastric cancer manuscript provides some thoughts as part of the discussion, this would be highly relevant for future work.

Reviewer #4

(Remarks to the Author)
Wen et al. revised their manuscript on microvesicles (MVs) in Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC). They found that viral infection coupled with extracellular vesicles plays a role in control of Hippo signaling, highlighting OLFM4-Hippo axis as a potential therapeutic target for EBV associated cancers. This Reviewer #4 raised several concerns for the originally submitted version, considering some unclear, confusing, or weak parts of the manuscript. The concerns have been addressed by the authors in this revision, but the authors are still encouraged to do additional experiments to polish their data.

Specific comment 1. The reviewer understands that recombinant EBV-EGFP virus was infected, and that GFP and EBNA-1 were confirmed to be expressed after the Akata treatment. Although infection rate of EBV in GES-1 and HGC-27 was not so low, approximately 20%, EBV-infected cells were not selected and the data was therefore not very clear. It should not be very difficult, and the authors are encouraged to sort EGFP-positive cells to select (or enrich) EBV-infected cells, and add some validation experiments using selected cells.

Specific comments 2-5. The authors have properly addressed all the concerns. Experiments using another cell line for MV treatment and shRNA expreiments are welcome addition. Overexpression of function-lost OLFM4 is also satisfactory, and their findings have been strengthened.

Version 2:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The authors have adequately addressed my concerns.

Reviewer #4

(Remarks to the Author)
In the revised manuscript, the authors have properly responded to this reviewer's request that they should sort EGFP-positive cells to select EBV-infected cells, and they have added supplementary figures to show the results.

A Point-by-point Response to the Reviewers' Comments

We thank the reviewers for their constructive comments. Accordingly, we have performed additional experiments and analyses, which mainly address the following raised issues:

1) The presence of OLFM4 in circulating MVs and the roles of intracellular OLFM4.

2) The reason to focus on OLFM4 in MVs.

3) EBV infection rate.

4) The functional relevance of OLFM4 by perturbation in EBV-infection models, and reason to use HEK293T cells.

5) Quality of the immunoblots and uncropped images.

Reviewer #1 (Remarks to the Author):

In this manuscript, authors identified olfactomedin 4 (OLFM4) containing large extracellular vesicles (EVs) induced by Epstin-Barr virus (EBV) infection in gastric cancers (GCs). Authors revealed that OLFM4 as a direct target gene of the cyclic GMP-AMP synthase-stimulator of interferon genes (cGAS-STING) pathway. Authors further demonstrated that **MV-containing OLFM4 as an extracellular inhibitory ligand of Hippo pathway**. The study is interesting, and the manuscript is well-written. There are some major and minor points to be addressed in order to improve the quality of study.

We appreciate the reviewer's the encouraging comments.

1) Fig. 3c, d, quality of STING immunoblot needs to be improved.

Following the reviewer's suggestion, we re-performed native polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting to improve the quality of STING immunoblotting in AGS-EBV cells and EBV-treated HGC-27 cells (**Fig. R1**). We found that EBV infection obviously increased the protein levels of OLFM4 (**Fig. R1a,b**). Moreover, EBV infection significantly enhanced STING oligomerization and promoted IRF3 phosphorylation and dimerization (**Fig. R1a,b**), indicating activation of the cGAS-STING signaling pathway upon EBV infection. We now incorporated these results into the revised manuscript.

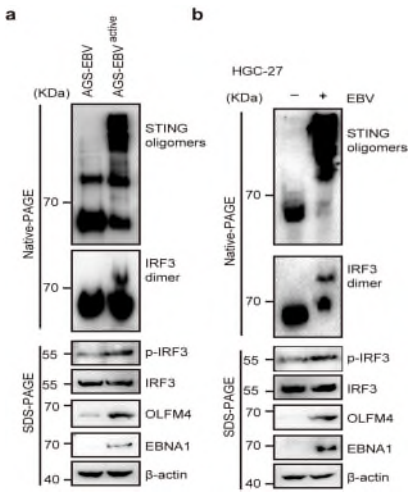


Fig. R1 EBV infection activates STING pathway. **a**, Immunoblotting for Native-PAGE and SDS-PAGE of lysates from AGS and EBV infected AGS (AGS-EBV) cells. **b**, Immunoblotting for Native-PAGE and SDS-PAGE of lysates from HGC-27 cells with or without EBV infection. AGS-EBV cells were treated with 0.5% Rabbit anti Human-IgG for 48 h. HGC-27 cells were infected with 10 MOI EBV for 48 h. All lanes were loaded with 50 μg of total protein.

2) Fig. 3f, OLFM4 immunoblot in response to RU.521 treatment was not clear.

We re-performed this experiment and found that STING agonist (cGAMP) increased the protein levels of OLFM4 in HGC27 cells, while cGAS inhibitor (RU.521) decreased OLFM4 expression (**Fig. R2**). We now updated this data in the revised manuscript.

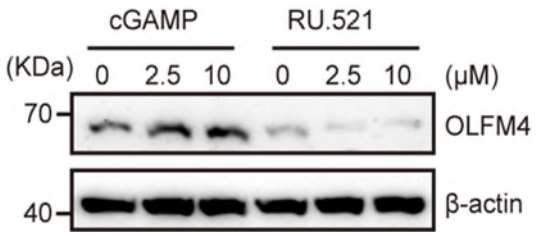


Fig. R2 cGAS-STING signaling regulates OLFM4 expression. Immunoblotting showing OLFM4 expression in HGC-27 cells treated with STING agonist (cGAMP) or cGAS inhibitor (RU.521) for 24h. All lanes were loaded with 50 μg of total protein.

3) Fig. 4, because the EVs usually are circulated in the blood, can OLFM4 be detected in the EBV positive GC patients' serum or blood samples?

The reviewer raised a very important question! To address this question, we collected EDTA-anticoagulated plasma and tissues samples from 7 EBV-positive (EBV^+) and 10 EBV-negative (EBV^-) GC patients (**Fig. R3a**). After isolation and characterization of MVs from plasma, we determined the protein levels of OLFM4 and found that OLFM4 was detectable in plasma MVs from almost all (6/7) EBV^+ GC patients (**Fig. R3a**). However, OLFML4 protein was only detectable in MVs from 3 out of 10 EBV^- GC patients (**Fig. R3a**). Importantly, OLFM4 levels were much higher in the MVs isolated from plasma and tissues of EBV^+ GC patients, compared to those of EBV^- GC patients (**Fig. R3b**). Together, these data suggest EBV-infected GC tissues tend to release OLFM4-containing MVs into peripheral blood, which might disturb systemic homeostasis during GC tumorigenesis.

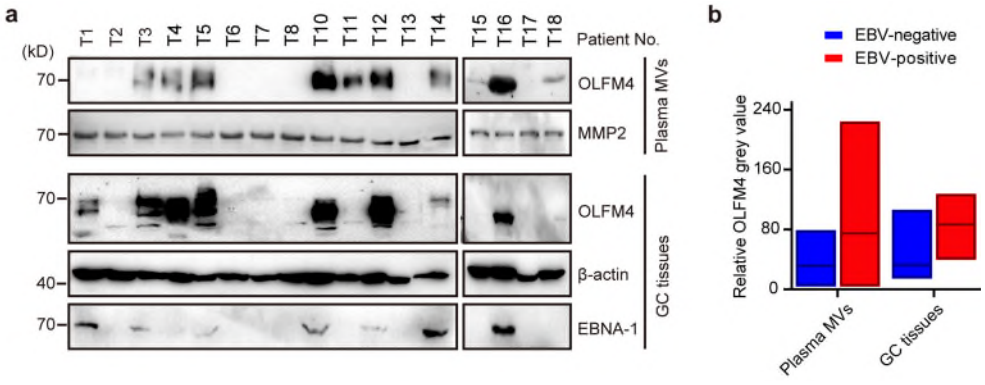


Fig. R3 Plasma-derived MVs from GC patients contain OLFM4. a, Immunoblotting showing OLFM4 expression in plasma MVs (upper) and tumor tissues (lower) from 7 EBV-positive (EBV^+) and 10 EBV-negative (EBV^-) GC patients. Total protein and MVs

were extracted from the 17 cardiac gastric cancer tissues for western blot analysis. For GC tissue, each lane loaded with 50 μg of total protein. For plasma MVs, each lane loaded 2 x 10^8 MV particles. **b**, Relative gray values showing the protein levels of OLFM4 in the purified MVs from the indicated samples.

4) Fig. 5, align with above comment, the effects of OLFM4 containing EVs on tumor formation needs to be detected by intravenous injection.

Agree! As mentioned above, we observed that EBV-infected GC tissues release OLFM4-containing MVs into peripheral blood (**Fig. R3**). To validate the effect of OLFM4-containing MVs in a context of systemic administration, we treated tumor-bearing nude mice with MVs by intravenous injection (**Fig. R4a**). We found that intravenous administration of OLFM4-containing MVs in nude mice increased tumor weight and promoted tumor progression (**Fig. R4b,c**). The phenotype here is less prominent when compared to those with intratumoral injection of OLFM4 containing MVs, most likely due to less MVs reaching the subcutaneous tumor. Meanwhile, we analyzed the transcription of YAP signature genes. Consistently, mRNA levels of CTGF and CYR61 were much higher in tumors from mice receiving OLFM4-containing MVs compared with those in mice receiving control MVs (**Fig. R4d**), indicating a pro-proliferative effect of OLFM4-containing MVs by intravenous injection.

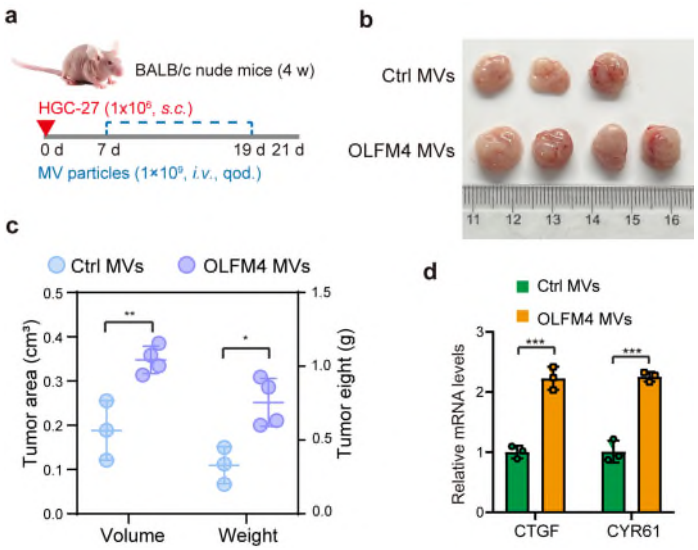


Fig. R4 OLFM4-containing MVs promotes GC growth. a, Schematic illustration of HGC-27 subcutaneous tumor model and intravenous MVs administration. Once tumors reached a size of 100 mm^3, mice were injected through tail vein with Ctrl or OLFM4 MVs (1×10^9 MV particles in 100 μl HEPES buffer) derived from HEK293T cells every other day. All mice were euthanized 21 days after cell inoculation. **b**, Representative images showing the effect of OLFM4 MVs on tumor formation by intravenous injection. **c**, Tumor area and

tumor weight in HGC-27 tumor-bearing mice after treatment with control or OLFM4-overexpressing MVs. **d**, Quantitative PCR detection of YAP target genes in subcutaneous tumors from mice intravenously injected with OLFM4 MVs. Bar graphs show mean +/− s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001.

5) Line 248, Authors have described:"5x106 HGC-27 cells were injected into nude mice.." Line 596, Authors have described:"HGC-27 cells (1x106 cells in 100 μl medium) were injected into flanks of 4-week-old male Balb/c nude mice.." the cell numbers in animal works need to be clarified.

In fact, we subcutaneously injected 1×10^6 HGC-27 cells into the right flanks of the nude mice. We now carefully went through our manuscript and corrected similar inconsistent description. Thanks!

6) According to Hippo-YAP regulation theory, YAP predominantly localizes in the nuclear under the low cell density condition. However, authors showed YAP extensively excluded from nuclear even in the sparse cell growth condition (Fig. 6i, top). Can author explain why this happened? Was intrinsic Hippo signaling impaired in this cell line?

We agree with the reviewer on the point of cell density-dependent YAP localization. We tend to believe that intrinsic Hippo signaling is intact in this cell line but the untypical observations might be due to culture condition. To address this issue, we reperformed the assay and provided representative images and quantification for YAP staining in ctrl MVs- or OLFM4 MVs-treated cells (**Fig. R5**). The results showed that OLFM4 MVs significantly promoted YAP nuclear translocation under high-density culture condition (**Fig. R5**). We now incorporated this data in our revised manuscript.

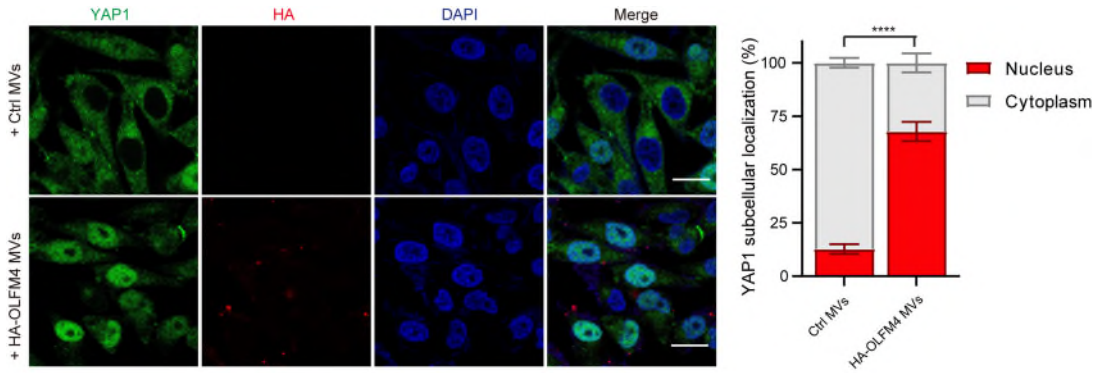


Fig. R5 OLFM4 MVs promotes YAP nuclear localization. Immunofluorescence analysis of YAP in control or OLFM4 MVs-treated HGC-27 cells. HGC-27 cells were grown to 80% confluency and then treated with 3 x 10^8 particles/ml HEK293T derived control or HA-OLFM4 MVs for 16 h. cells were washed and fixed for immunofluorescence analysis. OLFM4 was marked in red (anti-HA-tag). Quantification for YAP nuclear translocation was shown. Scale bars, 10 μm. Statistical analysis was performed using two-tailed unpaired Student's t-test. Bar graphs show mean +/− s.e.m. ****: p<0.0001.

Reviewer #2 (Remarks to the Author):

In this study, Wen et al. found that EBV infection activates cGAS-STING signaling to induce the expression of OLFM4, which can be secreted outside of the cells via MVs. OLFM4-carried MVs act on the cancer cells to inhibit the Hippo-Yap pathways, promoting cell proliferation and tumorigenesis. OLFM4 binds to FAT1 to abrogate its intracellular interaction with MST1, leading to enhanced YAP activation. The following concerns need to be addressed to improve the rigor of the study.

We appreciate the reviewer's encouraging and constructive comments.

Major concerns:

1. What cell types are infected by EBV in the TME? While the histology data showed that OLFM4 is highly expressed in EBV++ cancers, the cell types that are infected by EBV are unclear. Based on the cell culture experiments, it seems that both cancer cells and epithelial cells can be infected by EBV. Then, what is the source of the cells that produce OLFM4?

We understand the reviewer's concern about the type of EBV-infected cells that produce OLFM4 MVs. Actually, EBV is a human herpesvirus that exhibits strong infection tropism for B lymphocytes and efficiently immortalizes them *in vitro*[1]. After primary infection, EBV establishes the lifelong state of virus carrier. In this state, EBV can be detected in two different tissues, B lymphocytes and epithelial cells, and is potentially oncogenic for both cell types[2, 3]. According to our finding, both normal gastric epithelial cells (GES-1 cells) and gastric cancer cells are EBV-susceptible host cells (**Fig. R6a**). Moreover, EBV infection significantly promoted OLFM4 expression and secretion in these cells (**Fig. R6b**). Considering high proportion of tumor cells in TME, we thereby reasoned that cancer cells are the main cell sources that produce OLFM4 MVs. However, we cannot rule out the possibility that EBV may infect normal epithelial cells in EBV-associated gastritis, which could eventually lead to gastric cancer. Further studies such as single-cell proteomics are needed to exactly distinguish EBV infected and OLFM4 high expressed cell types.

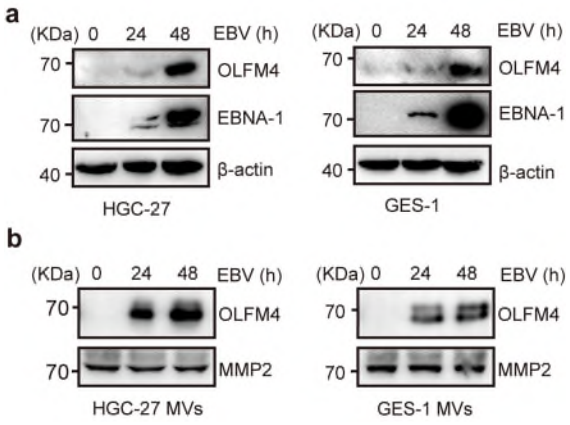


Fig. R6 EBV infection of both cancer cell and normal epithelial cell. **a**, Immunoblotting analysis of protein levels of OLFM4 and EBNA-1 in HGC-27 (left) and GES-1 (right) cells. Cells were treated with 10 MOI EBV for 0, 24, and 48 h. All lanes were loaded with 50 μg of total protein. **b**, Immunoblotting of OLFM4 levels of in the purified MVs isolated from EBV-infected HGC-27 (left) and GES-1 (right) cells. All lanes were loaded with 2×10^8 particles of total MV.

If cancer cells produce OLFM4 on their own, what are the roles of intracellular OLFM4 in cancer cell proliferation?

The reviewer raised an important issue! Here, we propose that EBV-infected gastric cancer cells secrete OLFM4-containing MVs to stimulate overgrowth of the neighboring cells that are not infected with EBV (**Fig. R7a**). As mentioned in the original manuscript, knockdown of OLFM4 (The knock down efficiency was confirmed by qPCR and western blotting) (**Fig. R7b**) obviously reduced cell proliferation and colony formation assay (**Fig. R7c,d**).

To further define the role of intracellular OLFM4 in cancer cell proliferation, we constructed a mutant OLFM4 lacking the N-terminal signal peptide (corresponding to amino acid residues 1-20) and termed as OLFM4 (DelSP). We confirmed in both AGS and HGC-27 cells that secretion of the OLFM4 (DelSP) mutant was disabled and therefore could not be detected in the purified MVs (**Fig. R7e,f**).

Subsequent colony formation (**Fig.R7g,h**) and sphere formation (**Fig.R7i,j**) assays in both HGC-27 and AGS cells suggested that overexpression of OLFM4 (DelSP) was still able to significantly promote cancer cell growth, suggesting that an important role of the intracellular OLFM4. In this regard, the specific mechanism of the intracellular OLFM4 remains to be clarified.

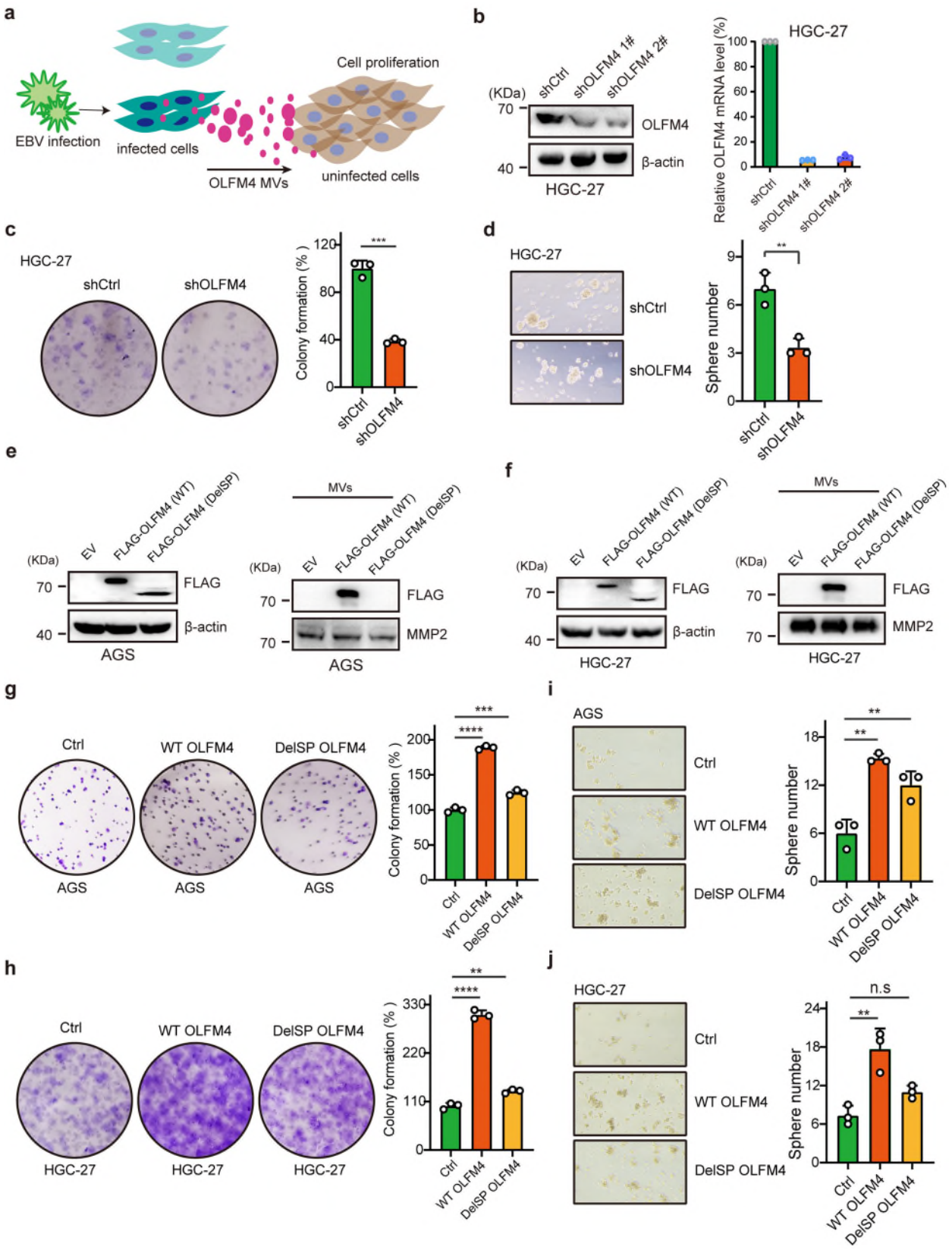


Fig. R7 Role of the intracellular OLFM4. a, Diagram. EBV-infected gastric cancer cells secrete OLFM4-containing MVs, which stimulate overgrowth of the neighboring cells that are not infected with EBV. **b**, Western blot and qPCR to confirm the knock down efficiency

of OLFM4 in HGC-27 cells. **c-d**, Representative images showing colony formation and sphere formation of OLFM4 knocked down HGC-27 cells. **e-f**, Western blot to detect the expression of wildtype and mutant OLFM4 (DelSP) in AGS and HGC-27 cells and related MVs. DelSP, deletion of the signal peptide (1-20 aa) of OLFM4. **g-h**, Representative images showing colony formation of AGS and HGC-27 cells overexpressing wildtype or mutant OLFM4. Three technique replicates were performed in each group. **i-j**, Representative image showing sphere formation of AGS and HGC-27 cells overexpressing wildtype or mutant OLFM4. Three technique replicates were performed in each group.

Can intracellular OLFM4 inhibit Hippo-YAP signaling?

To address this question, we employed the OLFM4 (DelSP) plasmid described above to assess the effect of intracellular OLFM4 on Hippo-YAP signaling and found that OLFM4 (DelSP) also significantly reduced the phosphorylation of both Mob1 and YAP1 in HGC-27 cells (**Fig. R8a**) and increased the mRNA levels of YAP target genes (**Fig. R8b**). We also observed that overexpression of OLFM4 inhibited the Hippo-YAP signaling, while knockdown of OLFM4 promoted the phosphorylation of YAP and MOB (**Fig. R8c,d, Original manuscript Extended data Fig.10**). Together, these results suggest that intracellular OLFM4 can inhibit Hippo signaling to promote YAP activity. We now incorporated these data into the revised manuscript.

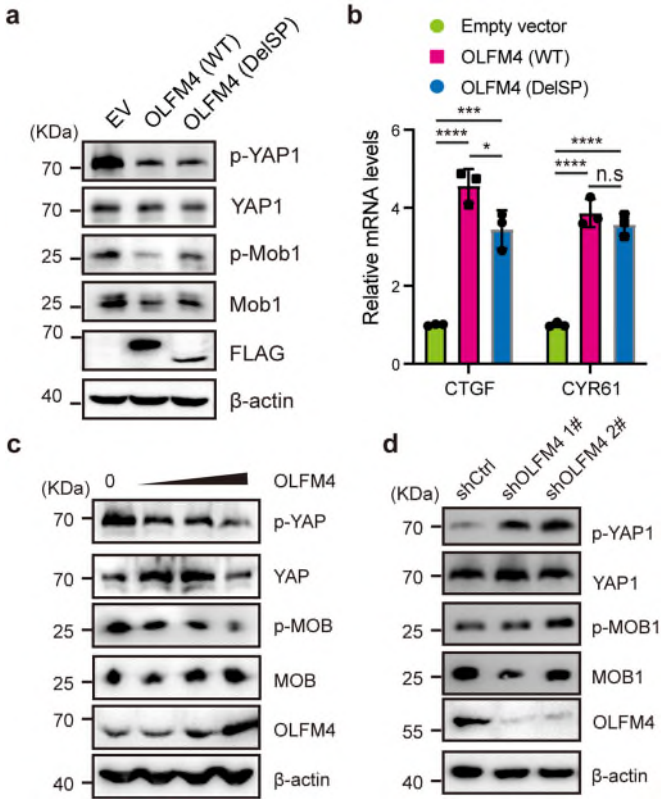


Fig. R8 Regulation of Hippo-YAP signaling by intracellular OLFM4. a,

Immunoblotting of p-Mob1, Mob1, p-YAP1 and YAP1 in HGC-27 cells transfected with empty vector (ctrl), wild-type OLFM4 or its signal peptide deletion mutant. **b**, QPCR analysis of YAP target genes (*CTGF* and *CYR61*) in the OLFM4 (DelSP)-expressing HGC-27 cells. **c,** Western blot showing overexpression of OLFM4 in HGC-27 cells transfected with 0, 2, 4, 8 μg pcDNA3.1-OLFM4 plasmids. After 48h incubation, cells were collected and lyzed for western blot. All lanes were loaded with 50 μg of total protein. **d,** Immunoblotting of p-Mob1, Mob1, p-YAP1 and YAP1 in OLFM4 knockdown HGC-27 cells.

2. Herpesvirus establishes a widespread infection in adult individuals. It is likely that EBV+ patients may also be infected by other herpesviruses. Can other herpesviruses induce OLFM4 expression? How can the authors rule out the impact of other herpesvirus infections on OLFM4 induction?

Following this reviewer's comments, we used herpes simplex virus 1 (HSV-1, KOS strain), an extensively studied herpesvirus to evaluate its impact on OLFM4 expression. In contrast to the effect of VSV infection (**Fig. R9a**) and EBV infection (**Fig R6**), we observed that HSV-1 infection inhibited OLFM4 protein expression in a dose-dependent manner (**Fig. R9b**). Moreover, HSV-1 infection significantly inhibited cGAS-STING antiviral immune responses (**Fig. R9c**), triggering the STING-dependent autophagy and degradation[4, 5]. Given the relationship between the cGAS-STING activation and OLFM4 expression, we speculate that OLFM4 expression is induced in a virus type-specific manner with a context of cGAS-STING activation.

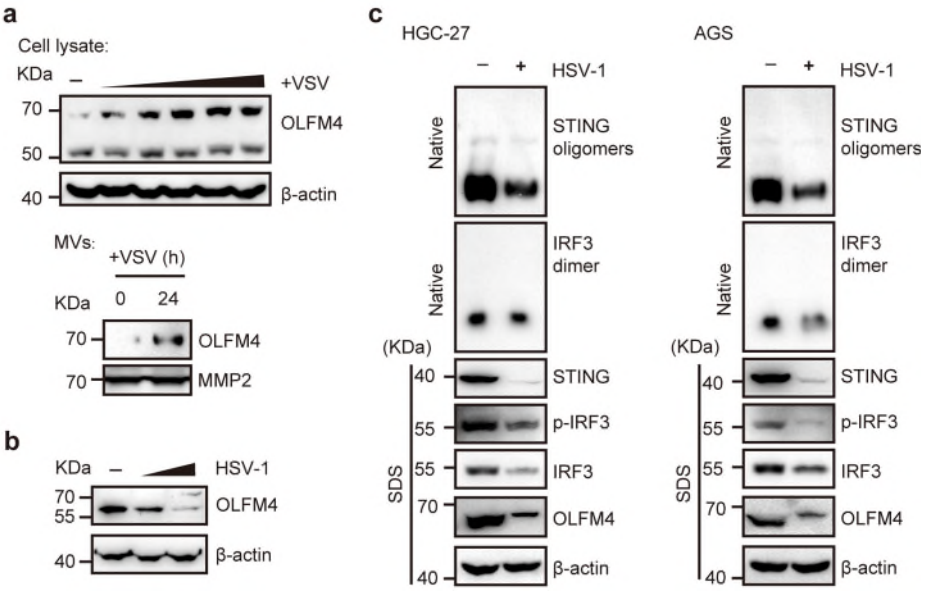


Fig. R9 Effect of HSV-1 on the expression of OLFM4. a, Immunoblotting showing the expression of OLFM4 in AGS cells after VSV infection (upper, 0, 0.5, 1.0, 2.5, 5.0, 10.0 MOI) or in the purified MVs isolated from VSV (10 MOI)-infected AGS cells (lower). AGS

cells were infected with Vero cell-producing VSV for 24h. **b,** Immunoblotting showing OLFM4 expression in HSV-1(0, 5, 10 MOI)-infected HGC-27 cells. **c,** Immunoblotting for Native-PAGE and SDS-PAGE of lysates showing the STING-related signaling in HSV-1 (10 MOI)-infected HGC-27 (left) and AGS (right) cells. All lanes were loaded with 50 μg of total protein.

3. In Fig. 3c-d, the molecular weight of STING seems incorrect. The STING oligomers and IRF3 dimers is unclear, and more convincing images should be provided.

Thank you for pointing out this issue. We re-performed native polyacrylamide gel electrophoresis (Native-PAGE) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting to improve the quality of STING and IRF3 immunoblotting (**Fig. R1**). The band of STING showed higher molecular weight in native-PAGE gels than SDS-PAGE gels as show in **Extended Data Fig. 3e** in the revised manuscript as described by other studies[6]. As expected, we found that EBV infection significantly enhanced STING oligomerization and promoted IRF3 phosphorylation and dimerization (**Fig. R1a,b**), evidencing activation of the cGAS-STING signaling pathway upon EBV infection. Of note, EBV infection obviously increased the protein levels of OLFM4 (**Fig. R1a,b**). We now incorporated these results into the revised manuscript.

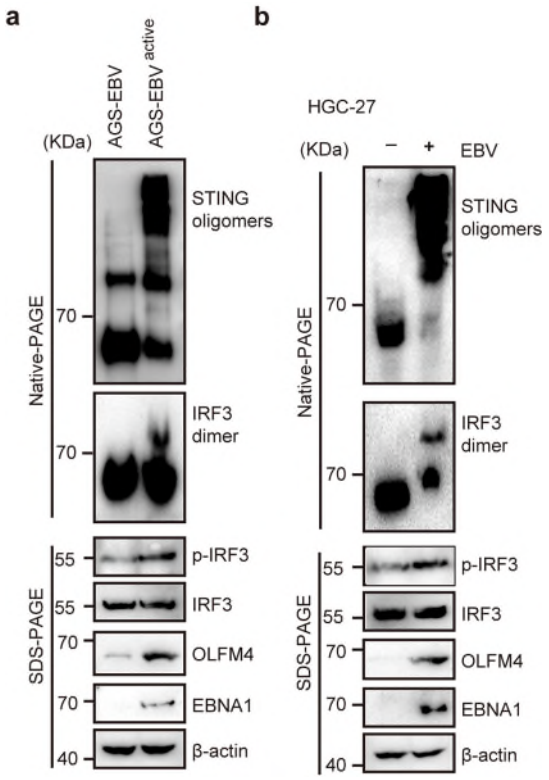


Fig. R1 EBV infection activates STING pathway. **a**, Immunoblotting for Native-PAGE and SDS-PAGE of lysates from AGS cells with or without EBV infection. AGS cells was cocultured with Akata-EBV to generate AGS-EBV cells. **b**, Immunoblotting for

Native-PAGE and SDS-PAGE of lysates from HGC-27 cells with or without EBV infection. HGC-27 cells were infected with 10 MOI EBV for 48 h.

4. In Fig. 3e, the OLFM4 expression only marginally decreased in STING knockdown cells. This might be caused by residual STING expression. To confirm that EBV-mediated OLFM4 induction is indeed STING-dependent, the authors should generate STING knockout cells and confirm OLFM4 expression in WT vs. STING KO cells in response to EBV infection.

Agree! To confirm that EBV-induced OLFM4 expression is STING-dependent, we created STING-knockout cells and examined OLFM4 levels by WB (**Fig. R10**). The result showed that EBV infection significantly elevated the protein levels of OLFM4 in AGS cells (**Fig. R10**). However, after knockout of STING, we found that EBV infection failed to upregulate OLFM4 expression (**Fig. R10**), indicating that EBV-induced OLFM4 expression is STING-dependent. We now incorporated these results into the revised manuscript.

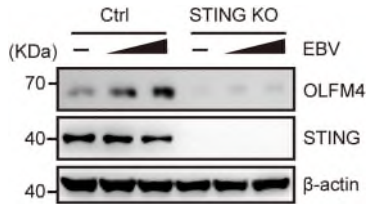


Fig. R10 EBV-mediated OLFM4 induction is STING-dependent. Immunoblotting showing the protein levels of OLFM4 level in wildtype (WT) and STING KO AGS cells upon EBV infection (MOI of 0, 5, 10) for 48 h. All lanes were loaded with 50 μg of total protein.

Minor concerns:

1. In Fig. 2A, could the author explain why GAPDH is present in the supernatant?

This is a keen-eyed reviewer. Our results showed that GAPDH was undetectable in the supernatants of HGC-27 and HEK293T cells (**Fig. R11a**). However, when it comes to tumor tissues, we used scissors to shear tumor tissues, which is very likely to cause partial release of intracellular contents. Thus, GAPDH, a highly abundant cytosolic protein, was detectable in the supernatants of the processed tumor samples (**Fig. R11b**).

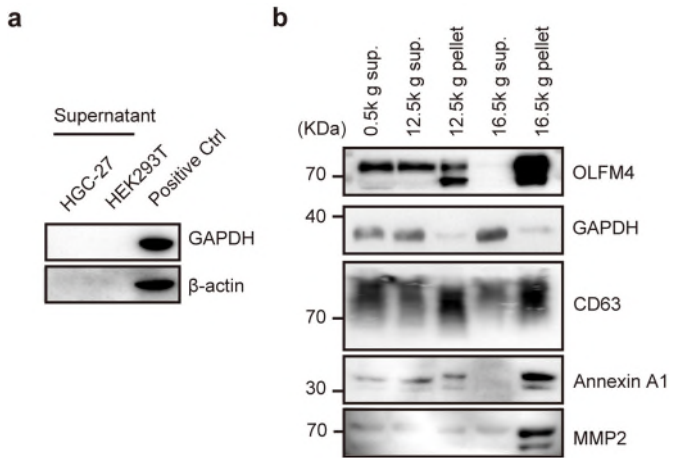


Fig. R11 Analysis of GAPDH in MVs. a, Immunoblotting of GAPDH in the supernatants of HGC-27 and HEK293T cells. Lysate from HEK293T cells was used as a positive control. **b**, Immunoblotting showing OLFM4 MVs and GAPDH from the GC tissues.

2. Statistical analysis is required for Fig. 5g.

We performed two-tailed unpaired student's *t*-test analysis to compare differences between groups (**Fig. R12**). We now added the related description in the figure legends.

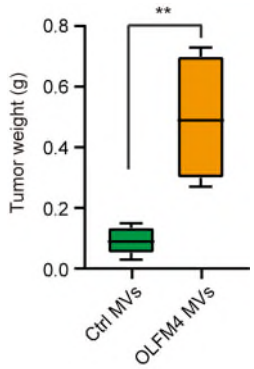


Fig. R12 OLFM4 MV treatment promotes tumor progression. HGC-27-induced tumor weight after treatment with OLFM4-containing (OLFM4) MV and control (Ctrl) MV (n = 6 mice/group). Data are presented as mean ± s.e.m, analyzed for significant differences by performing two-tailed, unpaired Student's *t*-tests. **, $p < 0.01$.

Reviewer #3 (Remarks to the Author):

In the manuscript by Wen et al. the authors describe how EBV in gastric cancer cells results in Olfactomedin 4 (OLFM4) positive microvesicle (MV) production. OLFM4 is activated by EBV through cGAS-STING signalling and this, in turn, promotes gastric cancer progression. Finally, the authors provide some data that OLFM4 inhibits HIPPO signal transduction. Overall, this is a multi-component cell biological study with some correlative clinical data. While interesting, I am a bit lost whether the MV component is connected to some other aspects of the study. That is to say, the authors mingle tissue positivity for OLFM4 and infer roles it might have (or the mere presence) in MVs. This disconnect is very obvious. Additionally, there is a large amount of important information and data withheld which does not increase the trust in the manuscript.

We thank the reviewer for the critical comments. In our study, we performed IHC and FISH analysis and confirmed the upregulation of OLFM levels in the tumor sample of EBV^+ GC patients (**Fig.4, original manuscript**). Moreover, we found that OLFM4 levels were much higher in the MVs isolated from tissues and plasma of EBV^+ GC patients, compared to those of EBV^- GC patients (**Fig. R13a-c**).

In addition, we also constructed a signal peptide deletion mutant of OLFM4, OLFM4 (DelSP), which lacks the N-terminal 1-20 aa and therefore could not be detected in the purified MVs (**Fig. R13d**). Of note, we found that these MVs isolated from the OLFM4 (DelSP)-transfected cells failed to activate YAP (**Fig. R13e,f**) and promote colony formation in HGC-27 cells (**Fig. R13g**).

Together, these data support a model (key findings of our study) in which EBV-infected gastric cancer cells secrete OLFM4-containing MVs to stimulate overgrowth of the neighboring cells that are not infected with EBV (**Fig. R13h**).

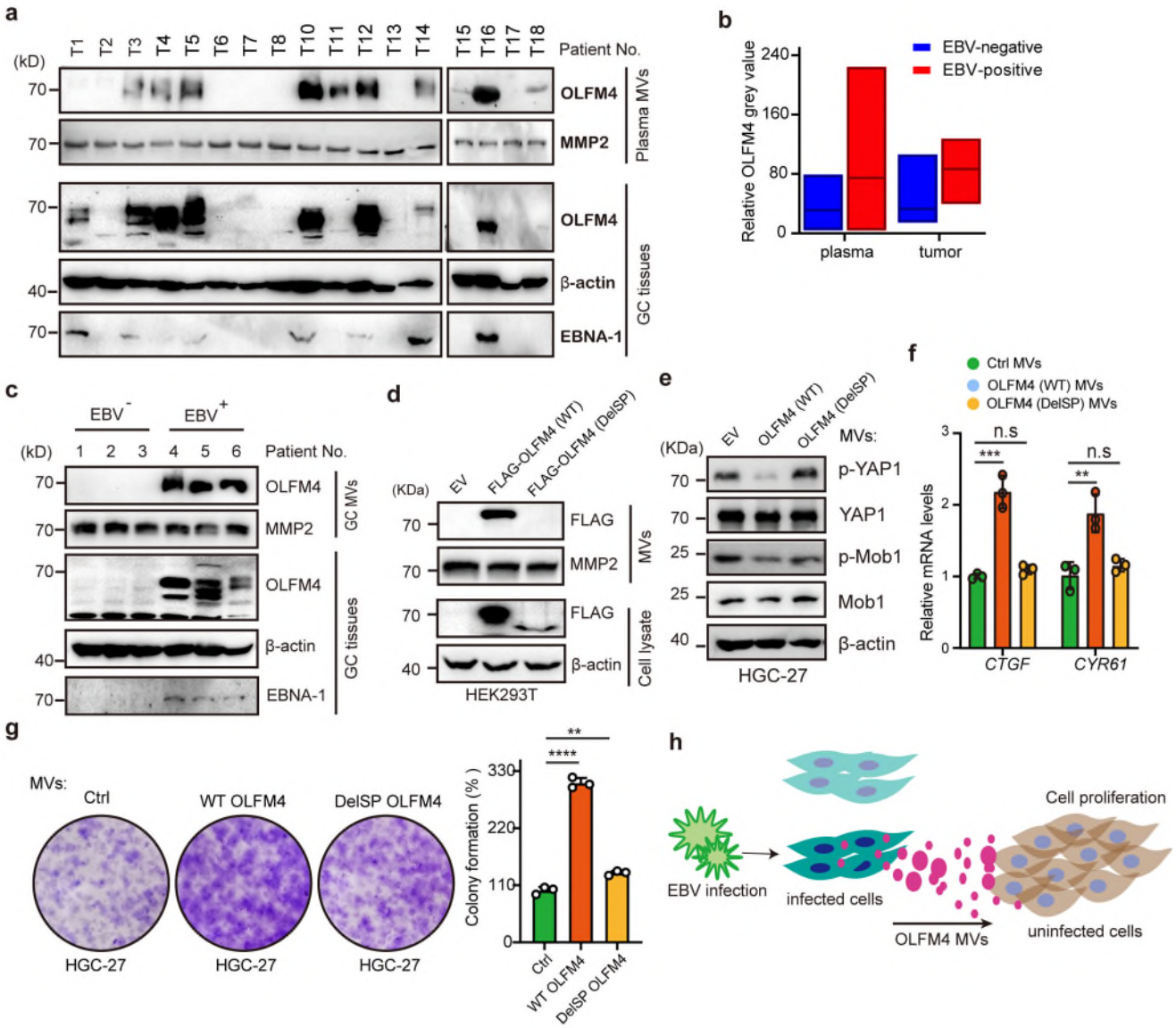


Fig. R13 Key findings of the current work. a, Immunoblotting showing OLFM4 expression in plasma MVs (upper) and tumor tissues (lower) from 7 EBV-positive (EBV^+) and 10 EBV-negative (EBV^-) GC patients. For GC tissue, each lane loaded with 50 μg of total protein. For plasma MVs, each lane loaded 2 x 10^8 MV particles. **b**, Relative gray values showing the protein levels of OLFM4 in the purified MVs from the indicated samples. **c**, Immunoblotting showing OLFM4 expression in tumor tissues and their MVs of GC patients. **d**, Immunoblotting of FLAG-OLFM4 (DelSP) in purified MVs from HEK293T**.** DelSP OLFM4, the signal peptide deletion mutant of OLFM4. All lanes loaded 2 x 10^8 particles for MV, or 50 μg of total protein for cell lysate. **e**, Immunoblotting of p-Mob1, Mob1, p-YAP1 and YAP1 in HGC-27 cells after pretreatment with empty vector (ctrl)-, wild-type OLFM4- or its deletion mutant MVs. **e**, QPCR analysis of YAP target genes (*CTGF* and *CYR61*) in HGC-27 cells after pretreatment with the indicated MVs. **f**, Colony formation of HGC-27 cells after pretreatment with the indicated MVs. **g**, Diagram. EBV-infected gastric cancer cells secrete OLFM4-containing MVs to stimulate overgrowth

of the neighboring cells that are not infected with EBV.

A few key points:

Methods: the method section is comprehensive, however, lacks all details to the specifics of the reagents used. For example, lot, catalogue, or version information is completely absent and needs to be added.

Thanks for pointing out this issue. We now provide more experimental details on reagents, including lottery, catalogue, or version informationin the revised manuscript (**Extend data table 8**).

All Fig.s/legends are missing the information on the number of technical and biological repeats completely. Please refrain from non-informative lines like, all lanes were loaded with the same amount of protein‘ but provide the specifics that whatever amount was used.

Agree! We now provided the related information in Figures and Figure legends in the revised manuscript. Thanks!

For MV specific analysis, why is same protein and not Same particle number used as reference? It is strongly suggested that in MV data, the same particle amount should be used, or at least referenced, as in ,so much protein is loaded in all lanes which equal x, y and z particles in the lanes, respectively.

Thanks for pointing out this issue. Indeed, we used “same protein” (μg) in MV analysis, which have been used in several classical MV studies[7, 8]. Notably, we used the same MVs ($1.5\sim3\times10^8$ particles/ml) in these analyses. We now added the related description in the method section.

Would the authors please provide all western images in the extended data in an uncropped version. Also additional high mag of TEM images (to show more than 1) and/or lower mag images should be provided to demonstrate the general appearance of the MVs

We now provide an uncropped version of all western images (**Source data**). We also provide more TEM images in the revised manuscript (**Fig. R14**).

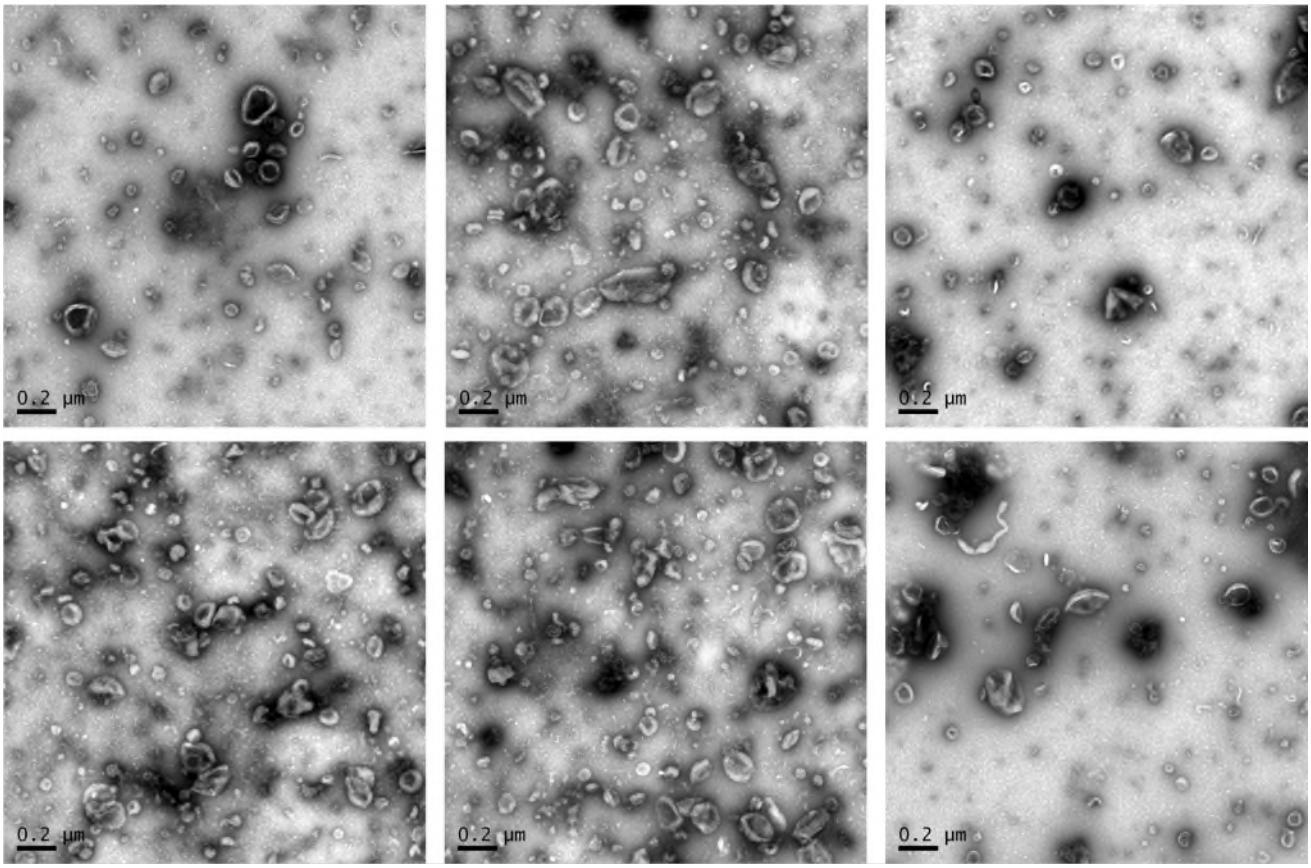


Fig. R14 TEM images of MVs isolated from GC tissues.

1e: The CD63 blot: how was this western done? Most CD63 antibodies require non-denaturing conditions, which are not described in the methods and the bands for CD63 do not look as these most commonly shown by authors for MV and other EV data.

We used a standard WB procedure for CD63 immunoblotting with its specific antibody (Santa Cruz，sc-5275)[9]. Following the reviewer's comment, we further performed native polyacrylamide gel electrophoresis (Native-PAGE) to detect CD63 levels in the MVs isolated from GC tissue. As expected, immunoblotting showed that CD63 protein was enriched in these MVs (**Fig. R15**). We now incorporated this result in the revised manuscript.

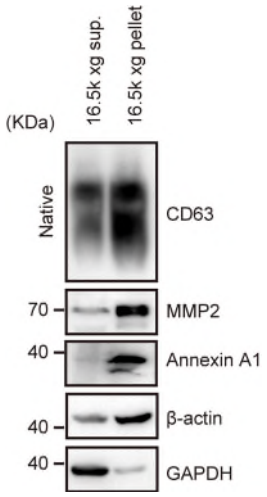


Fig. R15 Identification of purified MVs. Immunoblotting for Native-PAGE and SDS-PAGE of MVs lysates from GC tissue. The same MVs (2×10^8 particles/ml) were used in the immunoblotting analysis.

1g requires labels to indicate the protein identities shown.

Agree. We now labelled the identities of the upregulated proteins in the EBV$^+$ GC group (**Fig. R16**).

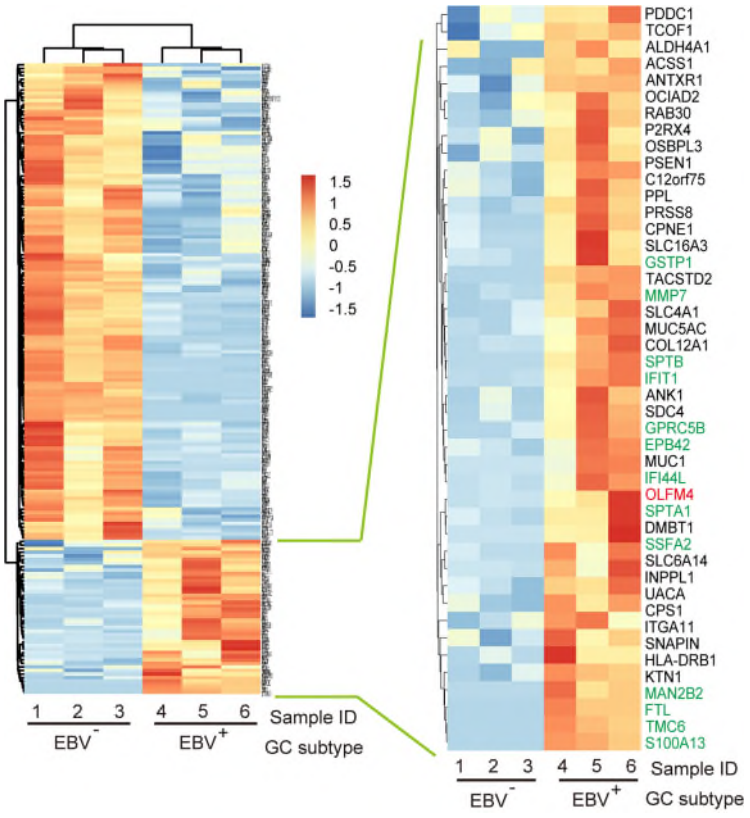
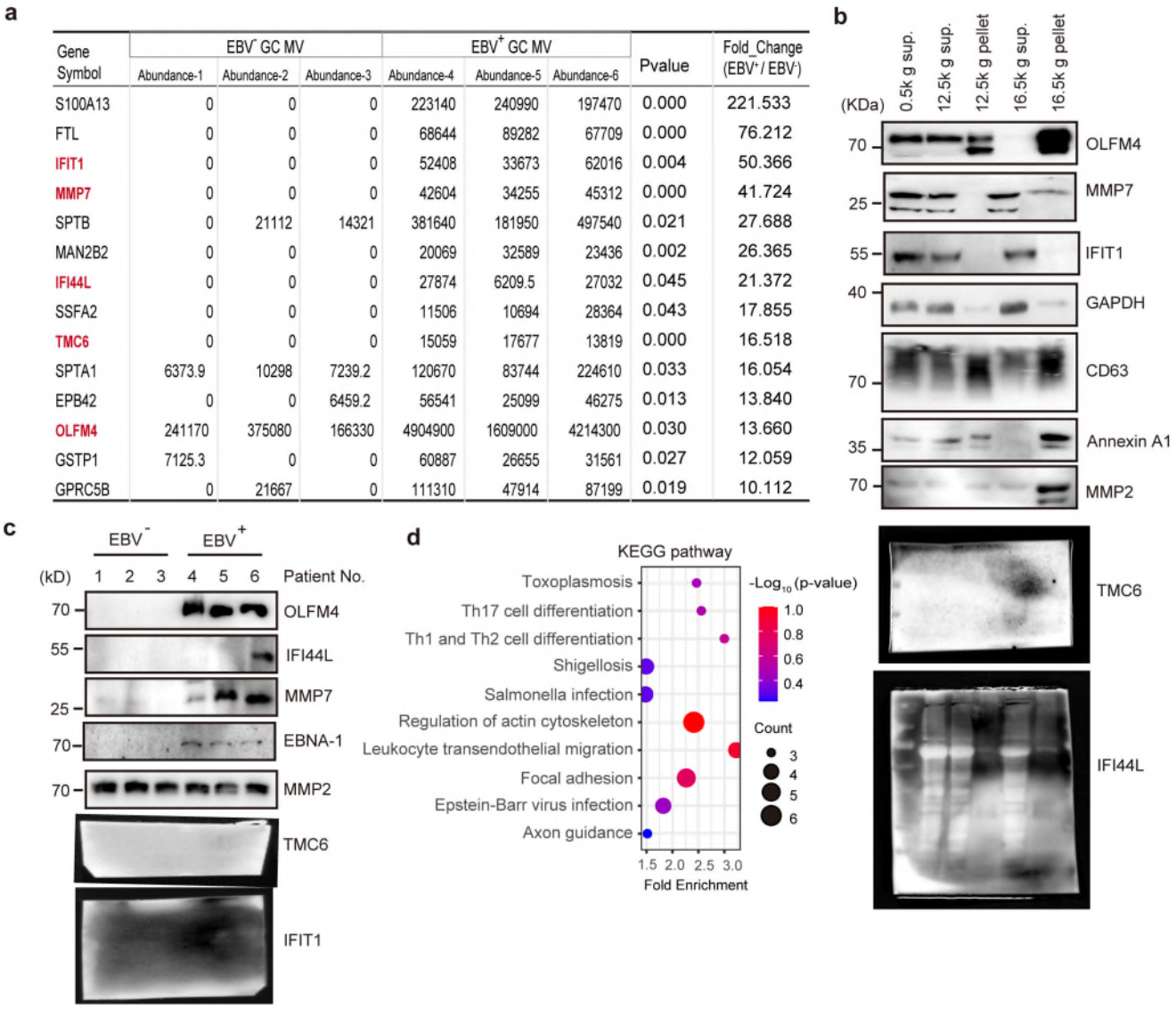


Fig. R16 Hierarchical clustering heatmap showing the differentially expressed proteins in MVs isolated from EBV$^-$ and EBV$^+$ GC tissues.

1f/g also require a method-independent validation of the MV data.

Following the reviewer's suggestion, we chose MMP7, OLFM4, TMC6, IFIT1 and IFI44L to validate our MV data (**Fig. R17a**). To this end, we performed western blotting to detect these significantly changed proteins in MVs of EBVnGC or EBVaGC. The western results showed that protein levels of MMP7, IFI44L, and OLFM4 were significantly higher in MVs of EBVaGC (**Fig. R17b**). Considering the heterogeneity of tumors, 3 replicates of MVs from GC cannot represent all. That said, the expression levels of OLFM4 in GC MVs seemed very high, findings consistent with the observation that the OLFM4 protein abundance was detected to be very high in MS data (**Fig. R17a**). Meanwhile, we could not detect TMC6 and IFIT1 in the MVs, likely due to their low abundance (nearly to the detection limit in MS data) (**Fig. R17b, c**).

Moreover, we found that some MVs markers, including MMP2, TSG101, RAB27B and RAB27A, could be identified (**Extended data table 1, original manuscript**). Further KEGG pathway analysis of the differentially expressed proteins showed that EBV infection-associated pathway was also enriched (**Fig. R17d, original manuscript Fig. 1h**).



Gene Symbol	EBV⁻ GC MV			EBV⁺ GC MV			Pvalue	Fold_Change (EBV⁺/ EBV⁻)
	Abundance-1	Abundance-2	Abundance-3	Abundance-4	Abundance-5	Abundance-6		
S100A13	0	0	0	223140	240990	197470	0.000	221.533
FTL	0	0	0	68644	89282	67709	0.000	76.212
IFIT1	0	0	0	52408	33673	62016	0.004	50.366
MMP7	0	0	0	42604	34255	45312	0.000	41.724
SPTB	0	21112	14321	381640	181950	497540	0.021	27.688
MAN2B2	0	0	0	20069	32589	23436	0.002	26.365
IFI44L	0	0	0	27874	6209.5	27032	0.045	21.372
SSFA2	0	0	0	11506	10694	28364	0.043	17.855
TMC6	0	0	0	15059	17677	13819	0.000	16.518
SPTA1	6373.9	10298	7239.2	120670	83744	224610	0.033	16.054
EPB42	0	0	6459.2	56541	25099	46275	0.013	13.840
OLFM4	241170	375080	166330	4904900	1609000	4214300	0.030	13.660
GSTP1	7125.3	0	0	60887	26655	31561	0.027	12.059
GPRC5B	0	21667	0	111310	47914	87199	0.019	10.112

Fig. R17 Validation of the MV data. a, Upregulated protein in the MVs of EBV-infected gastric cancer identified by mass spectrometry. **b**, Immunoblotting showing the levels of IFIT44L, MMP7, and OLFM4 in GC-drived MVs. **c**, KEGG pathway analysis for the 176 differentially expressed proteins.

1f indicates that MMP7 is even more promising candidate than OLFM4 on MVs and if the work focuses on the MVs as secretion pathway and mediator of the HIPPO pathway functions as per later, this should be assessed.

Agree! Indeed, we observed that EBV infection significantly stimulated intracellular MMP7 expression (**Fig. R18a**) and its MVs secretion (**Fig. R18b**). Following the reviewer's comments, we assessed the effect of MMP7 overexpressing MVs on the Hippo pathway. We observed no significant changes in the levels of p-YAP1, YAP1, p-Mob1 and Mob1, as well as YAP target genes, in the MMP7-overexpressing MVs-treated AGS cells (**Fig. R18c, d**). Given the previous

report that YAP signaling upregulates MMP7 expression and promote cell adhesion and migration[10-12], we speculate that OLFM4-overexpressing MVs activate YAP signaling, which may in turn stimulate MMP7 expression and secretion.

Except that, it is already reported that MMP7 could be induced by EBV, plays important roles in cell invasion ability of gastric cancer, and the MMP7 protein level in serum-derived exosomes have already reported as a well diagnostic biomarker for gastric cancer. As shown in **Fig. R17 b**, more MMP7 are left in 1,6500g supernatant fraction which contains exosomes. Thus we believe MMP7 is not the major component in EBV associated MVs in GC.

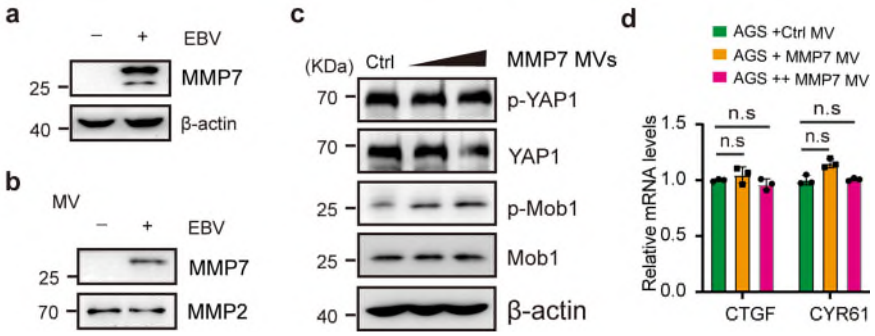


Fig. R18 Effects of MMP7 MVs on the Hippo signaling. a, Immunoblotting showing the protein levels of MMP7 with its specific antibody (1:2000, ab232737, Abcam) in GES-1 cells infected with EBV for 48 h. **b,** Immunoblotting showing the protein levels of MMP7 in MVs isolated from EBV-infected GES-1 cells. The same MVs (2×10^8 particles/ml) were used in the immunoblotting analysis. **c**, Immunoblotting showing the levels of p-YAP1, YAP1, p-Mob1 and Mob1 in AGS cells after treatment with MMP7-overexpressing MVs (1.5, 3×10^8 particles/ml) derived from HEK293T. **d**, Quantitative PCR analysis of YAP target genes in MMP7 MV- treated AGS cells.

1i: Why is a proteomic assessment of MV content shortlisted by gene expression changes in cancer samples? I cannot understand the reasoning as several EV publications show that the correlation between cell/tissue and EV/MV abundance of proteins (and RNA) cannot be assumed-which exactly is done here. The argument that the MMP7 levels are lower in cells (1J) is therefore confusing: as mentioned above, is the investigation on the role of OLFM4 in MVs or cells or both? No data is provided to demonstrate any correlations between cell and MV levels for OLFM4, MMP9 or any of the other shortlisted hits. Could it be that MMP7 or other MV hits having a log-scale higher fold change could be at more abundance, and therefore at least as important, to MV functions in EBV GC than OLFM4?

We understand this reviewer's concern about the direct correlation between intracellular OLFM4 and OLFM4-containing MVs. It is well known that EBV infection leads to the secretion of EV/MVs that carry a variety of cargoes, including RNAs, proteins, lipids and DNA, which could further promote cancer progression by

stimulate proliferation of recipient cells[13-15]. As shown in **Fig. R13c**, we observed an upregulation of OLFM4 in both GC tissue and GC-derived MVs from EBV-positive patients, hinting a positive correlation of OLFM4 levels between GC tissues and GC-derived MVs. Moreover, we observed that EBV infection significantly enhanced intracellular expression and MV secretion of OLFM4 (**Fig. 2d,e,g, original manuscript**) and MMP7 (**Fig. R18a,b**). As mentioned in the original manuscript, we found that treatment with OLFM4 MVs but not MMP7 MVs induced the activation of YAP (**Fig. R18c,d**). Furthermore, MMP7 MVs did not seem to affect colony formation (**Fig. R19a,b**) and sphere formation (**Fig. R19c,d**). Thus, we subsequently focused on OLFM4 MVs in GC progression.

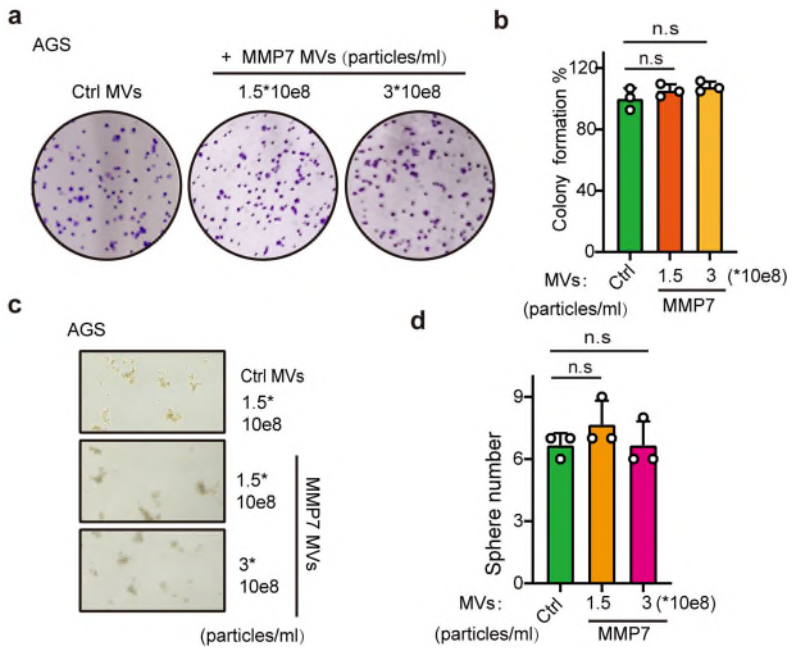


Fig. R19 Effect of MMP7 MVs on GC growth. a, Representative images showing colony formation ability of MMP7 MV-treated AGS cells. **b**, Quantification for colony formation assay. **c**, Representative images showing sphere formation ability of MMP7 MV-treated AGS cells. **d**, Quantification for colony formation assay. Three technical replicates were performed in each group. Bar graphs show mean +/− s.e.m. n.s., not significant.

A general question to the data in Fig1 and elsewhere: EV isolation protocols, both for small and large EVs, could, in principle, co-isolate virus particle. Have the authors evaluated if EBV GC cells or tissues either secrete EBV (and it is a co-isolate/contaminant) or whether it is contained within MVs? Are the recipient cells in later experiments EBNA positive after MV administration of EBV GC cells?

Extracellular vesicles (EVs) released by tumor cells function as a unique form of intercellular communication that can promote cell growth and survival, help shape

the tumor microenvironment and increase invasive and metastatic activity[16-18]. There are two major classes of EVs, microvesicles (MVs) and exosomes, and they differ in how they are formed. MVs are generated by the outward budding and fission of the plasma membrane. On the other hand, exosomes are derived as multivesicular bodies fused with plasma membrane and release their contents[19]. Because our centrifugation rate for MV extraction is 16500g, which is lower than the speed used for virus purification (usually need ultrahigh speed and longer time). Indeed, we detected no EBNA1 signal in either purified MVs from clinical GC tissues (**Fig. 20a**) or cells receiving MVs derived from EBV GC cells (**Fig. 20b**). That said, we cannot rule out the possibility of low abundance of EBV particles in the purified MVs.

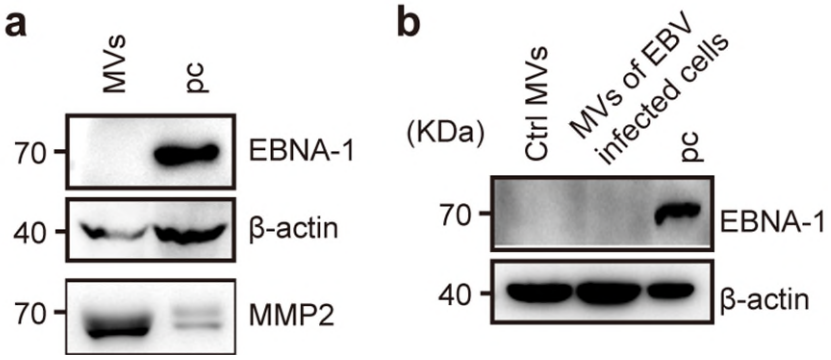


Fig. R20 Detection for EBV particles in MVs. a, Immunoblotting of EBNA-1 in the purified MVs from tumor tissues of an EBV-positive GC patient. Positive control (pc), EBV-infected tumor tissues. Lane 1 loaded 2 x 10^8 MV particles. lane 2 loaded with 50 μg of total protein. **b,** Immunoblotting of EBNA-1 in AGS cells receiving control MVs or MVs derived from EBV-infected AGS cells. MVs (3×10^8 particles /ml) from EBV-treated AGS cells were collected and used to treat uninfected AGS cells for 24h. Positive control (pc), EBV-infected (10 MOI, 48h) AGS cells. All lanes loaded with 50 μg of total protein.

Fig2a/b: This experiment should a) be shown for more than 1 example and b) for EBV negative GC tissues. Some data is okay to be added to extended data. In 2a, could the authors please show the same MV markers/cell markers as in Fig1e

Agree. As mentioned earlier, we analyzed the expression of OLFM4 in MVs and tumor tissues from GC patients and found an upregulation of OLFM4 levels in both GC tissue and GC-derived MVs from EBV-positive patients (**Fig. R13a-c**). Consistently, we applied TEM and observed that OLFM4 was detectable in the MVs from EBV-positive tumor tissues while we failed to detect any positive signal in MVs of EBV-negative GC (**Fig. R21a**). Regarding Fig. 2a, we now provided MV markers (**Fig. R21b**). We incorporated these data into the revised manuscript.

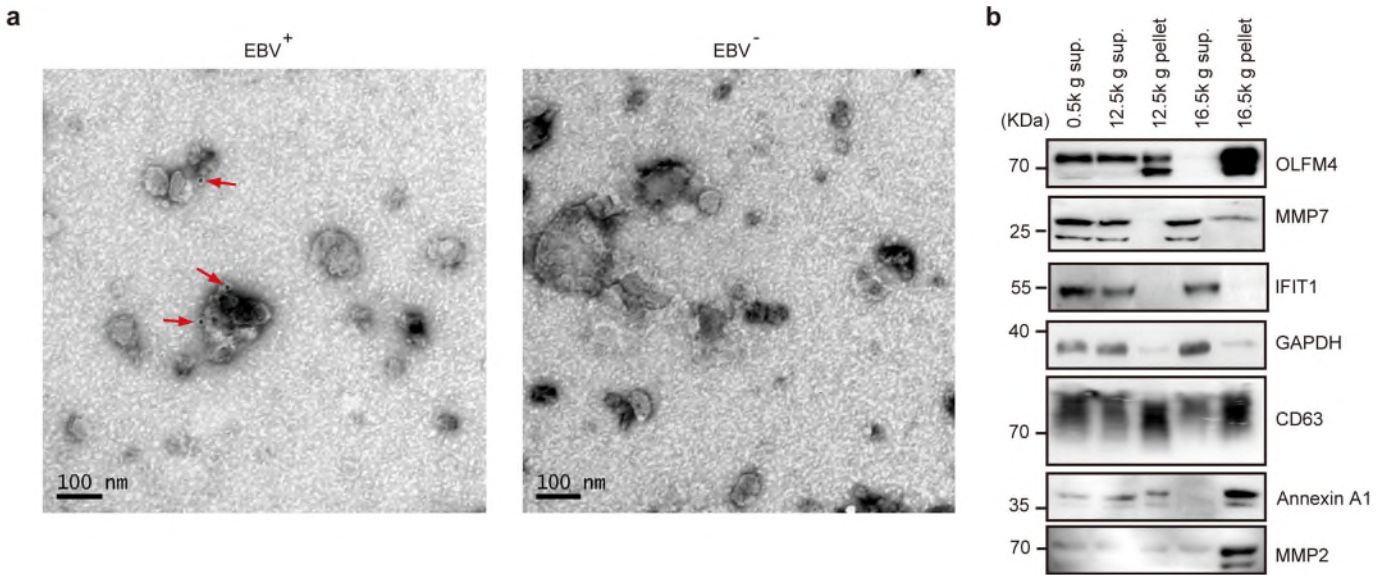


Fig. R21 OLFM4 is enriched in MVs isolated from EBV-positive GC tissues. **a,** Representative TEM images showing the immunogold-labelled OLFM4 in GC-derived MVs. Arrowheads indicate 10 nm gold particles. Scale bar, 100 nm. **b,** Immunoblotting showing the efficiency of MVs isolated from the tumor tissues of GC patient.

In Fig2, the abundance of MMP7 and other proteins with higher abundance in MVs than OLFM4 should be shown.

We now provided the MS data on the MMP7 and other proteins with higher fold-change in MVs than OLFM4 (**Fig. R17a**). Note that the abundances of MMP7 and other proteins with higher fold-change are much lower than OLFM4 (**Fig. R17a**). In addition, we also examined the protein levels of IFIT1, IFI44L, TMC6, MMP7, and OLFM4 in GC-drived MVs. Immunoblotting showed that IFI44L, MMP7 and OLFM4 were readily detected in MVs from EBV positive but not MVs from EBV GC tissues (**Fig. R17c**). Meanwhile, TMC6 and IFIT1 were undetectable in MVs from both EBV negative and EBV positive GC tissues (**Fig. R17b,c**).

a

Gene Symbol	EBV⁻ GC MV			EBV⁺ GC MV			Pvalue	Fold_Change (EBV⁺/ EBV⁻)
	Abundance-1	Abundance-2	Abundance-3	Abundance-4	Abundance-5	Abundance-6		
S100A13	0	0	0	223140	240990	197470	0.000	221.533
FTL	0	0	0	68644	89282	67709	0.000	76.212
IFIT1	0	0	0	52408	33673	62016	0.004	50.366
MMP7	0	0	0	42604	34255	45312	0.000	41.724
SPTB	0	21112	14321	381640	181950	497540	0.021	27.688
MAN2B2	0	0	0	20069	32589	23436	0.002	26.365
IFI44L	0	0	0	27874	6209.5	27032	0.045	21.372
SSFA2	0	0	0	11506	10694	28364	0.043	17.855
TMC6	0	0	0	15059	17677	13819	0.000	16.518
SPTA1	6373.9	10298	7239.2	120670	83744	224610	0.033	16.054
EPB42	0	0	6459.2	56541	25099	46275	0.013	13.840
OLFM4	241170	375080	166330	4904900	1609000	4214300	0.030	13.660
GSTP1	7125.3	0	0	60887	26655	31561	0.027	12.059
GPRC5B	0	21667	0	111310	47914	87199	0.019	10.112

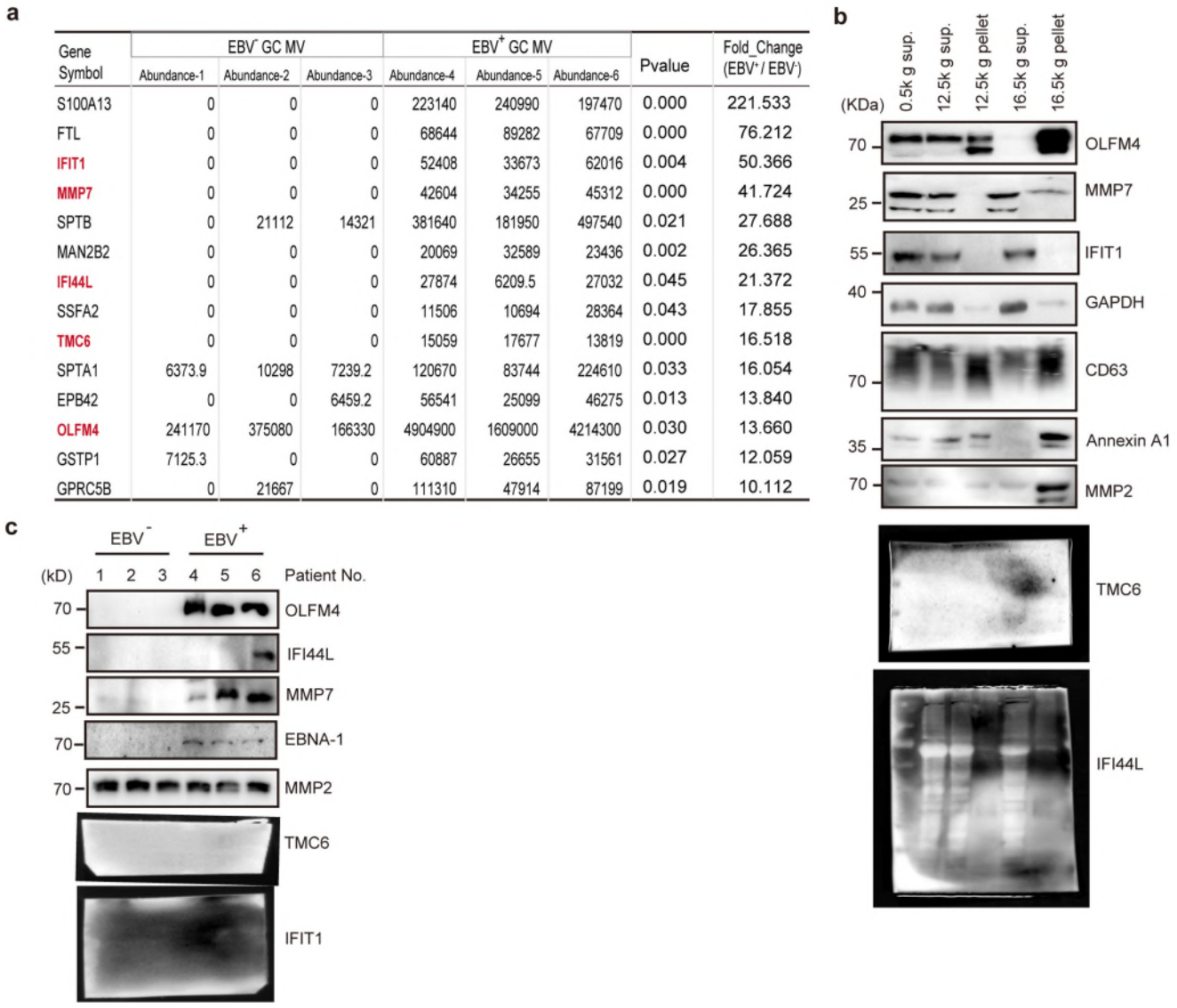


Fig. R17 Validation of the MV data. a, Upregulated proteins in MVs of EBV-infected GC identified by mass spectrometry. **b**, Immunoblotting showing the levels of indicated proteins in GC-derived MVs.

Fig2/text: An explanation for the non-GC specialist reader about the characteristics of HGC-27, GES-1 and other lines should be included.

Thanks for reminding us. We now provided the related description in the revised manuscript. "GES-1 is a normal gastric mucosal cell line. AGS is a GC cell line with a medium differentiation level. HGC-27 is a GC cell line obtained from the metastatic lymph node of a GC patient diagnosed histologically as undifferentiated carcinoma."

Fig3: Can EBNA in recipient cells or EBV in MV isolates be detected?

As mentioned above, we failed to detect EBNA1 signal in either purified MVs (**Fig. 20a**) or MVs-treated cells (**Fig. 20b**). This might be due to the low-abundance of

EBV particles in the purified MVs. It is also likely that the centrifugal force and duration time for extracting MVs (1,6500g) are not sufficient to precipitate the virus particle (usually need ultrahigh speed centrifugation and longer time).

Fig4: I still cannot understand the constructed relationship between OLFM4 on MVs and for example this tissue/patient data. Also, all MVs were either tissue or cell line derived. Do the authors suggest that the MV function, and therefore the OLFM4-MVs, only act in the local, immediate environment of the producing cell?

To address the reviewer's question, we detected OLFM4 expression in the local cancer tissues, as well as in the blood samples in the revised manuscript (**Fig. 22a-c**). We found that OLFM4-MVs not only exist in the local environment, but also can be delivered in the blood.

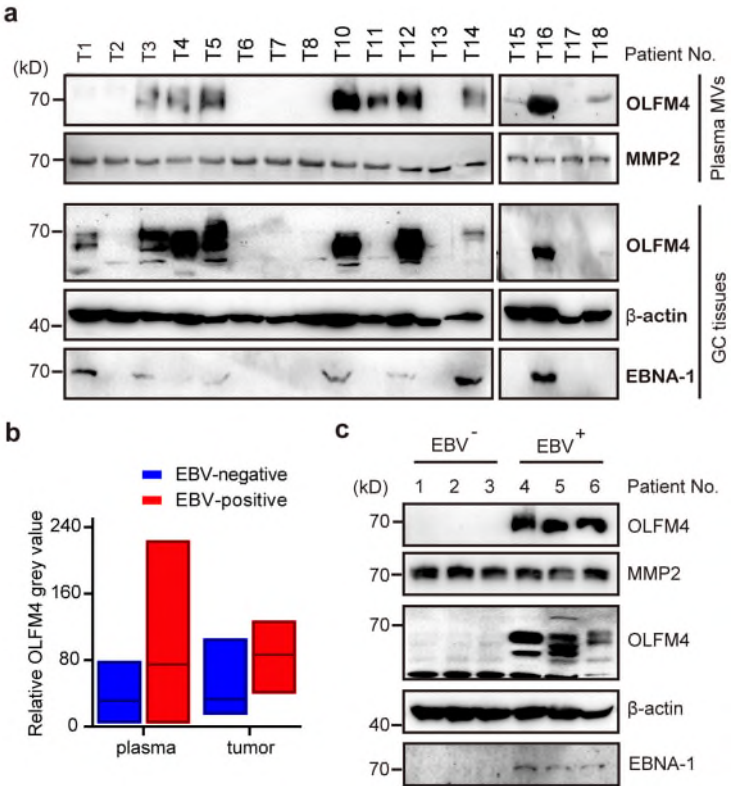


Fig. R22 EBV-infected GCs tend to secrete OLFM4-MVs. a, Immunoblotting showing OLFM4 expression in plasma MVs (upper) and tumor tissues (lower) from 7 EBV-positive (EBV^+) and 10 EBV-negative (EBV^-) GC patients. For GC tissue, each lane loaded with 50 μg of total protein. For plasma MVs, each lane loaded 2 x 10^8 MV particles. **b**, Relative gray values showing the protein levels of OLFM4 in the purified MVs from the indicated samples. **c**, Immunoblotting showing OLFM4 expression in tumor tissues and their MVs of GC patients. For GC tissue, each lane loaded with 50 μg of total protein. For plasma MVs, each lane loaded 2 x 10^8 MV particles.

Can OLFM4-MVs be found in the draining lymp nodes or the blood? For this figure, what are the OLFM4 levels on MVs in the tissues?

As described earlier, we collected EDTA-anticoagulated plasma and tissues samples from 7 EBV^+ and 10 EBV^- GC patients (**Fig. R13a**). After isolation and characterization of MVs from plasma, we determined the protein level of OLFM4 and found that OLFM4 was detectable in plasma MVs from all EBV^+ GC patients (**Fig. R13b**). However, OLFML4 protein was only detectable in MVs from 3 out of 10 EBV^- GC patients (**Fig. R3b**). These data suggest that EBV-infected GC tissues tend to release OLFM4-containing MVs into peripheral blood.

Fig5/extended data 2: Why are HEK293T cells used to overexpress OLFM4? What is the use? The explanation is absent and I am left wondering if this data was just ‚there' and put in to use it, or if there is any meaning to it. The only conclusion is that the 293 derived MVs are used in Fig5a, c, e, f, g. If this is the case, this is even more concerning as now the conclusion is that OLFM4 regardless of reason might have an effect exclusively (?) on GC cells. Are the MVs associating with the HGC27 cells, are they taken up, how many MVs are used/cell, how often repeated.

We are sorry for having not clearly described the use of MVs from HEK293T. Actually, HEK293T cells have been widely used as EV producer cells due to their inherent rapid proliferation, high EV yield, and ease of genetic manipulation[20-23]. The stem cells or metabolic active cells are thought to internalize significantly more HEK293T EVs than terminally differentiated cells[20]. Theoretically, as most EVs under evaluation are derived from human cells, they may elicit immune responses in mice. However, some studies have assessed the immune response and toxicity in mice, and found that HEK293T EVs showed low toxicity, and minimal changes in immune markers[20, 24].

Here, we used HEK293T cells to generate MVs aiming to exclude other potential pro-proliferative factors possibly present in GC-derived MVs. For example, the extracellular vesicles could carry a number of herpesvirus-associated proteins, including LMP1, EGFR and PI3K, from the host cells to the recipient cells to accelerate cell growth[13-15]. Thus, after excluding these carcinogenic factors from tumor cells, we were able to provide solid evidence for the function of OLFM4 MVs during GC progression.

In original manuscript Figure 7 (**Fig R23**), we found that OLFM4-containing MVs localized on the plasma membrane of recipient cells. We used 1.5 x 10^8, 3 x 10^8 particles/ml HEK293T derived OLFM4 in Fig 5. The culture medium containing OLFM4 MVs was replaced every other day. 3 replicates were performed in each group. We now added the detailed information in the revised manuscript.

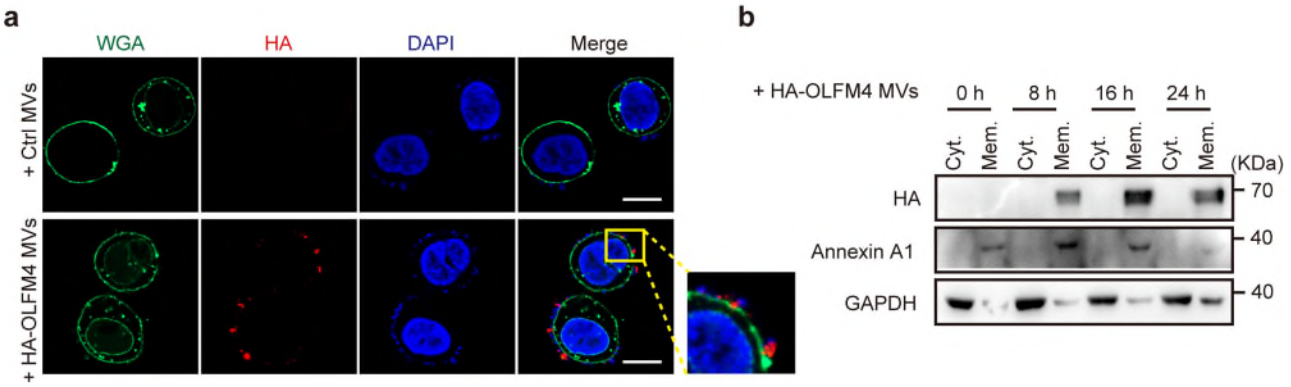


Fig R23 OLFM4 MVs localized on the plasma membrane of recipient cells. a, Immunofluorescence detection of MV-carried OLFM4 on recipient cells. AGS cells were treated with HA-OLFM4-overexpressing MVs (3×10^8 particles/ml) generated by HEK293T for 24 h. OLFM4 was labelled with anti-HA (red). Plasma membranes were stained by Wheat germ agglutinin (WGA, green). Scale bars, 10 μm. (b). Fractionation and western blot analysis of MV-carried OLFM4 in AGS cells. 3×10^8 particles/ml of MV derived from HEK293T cells were used for AGS cell treatment. Annexin A1 and GAPDH were used as markers for membrane and cytoplasm, respectively. Cytoplasm, Cyt.; Membrane, Mem.

5b, d: What was done here? WT HGC cells (WITH OLFM4 MVs) were treated with MVs derived from knockdown cells. First of all, there is no data anywhere showing the efficacy of the cellular knockdown, neither the effect of MV levels.

Thank you for pointing out this issue. Yes, we intended to evaluate the effect of externally added OLFM4 MVs on HGC-27 cell growth. To this end, we used HEK293T cells to produce MVs. By knockdown OLFM4 in the MV producer cells, we wanted to examine the role of OLFM4 in MV regulation of HGC-27 cell growth. Meanwhile, we performed immunoblotting and QPCR analysis to confirm the knockdown efficiency of OLFM4 in HEK293T cells (**Fig. R24**).

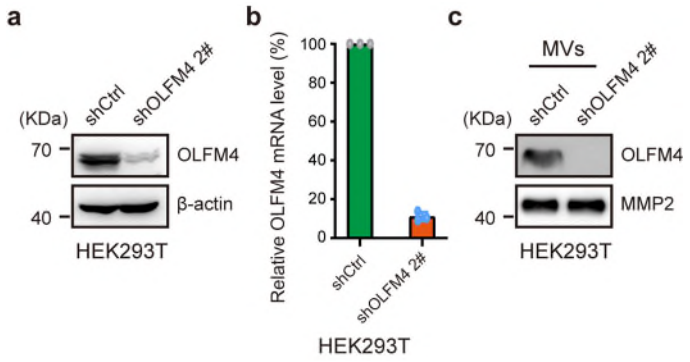


Fig R24 knockdown efficiency of OLFM4 by its specific shRNAs. a, Immunoblotting showing the levels of intracelluar OLFM4 in OLFM4-knockdown HEK293T cells. Scramble shRNA was used as a control. **b**, QPCR assay showing the mRNA levels of OLFM4 in

OLFM4-knockdown cells. **c,** Immunoblotting showing the levels of OLFM4 in MVs of OLFM4-knockdown HEK293T cells. All lanes loaded 50 μg of total protein.

Then, the use of a reduced OLFM4-MV (assuming this might be the case) is impacting on cells producing OLFM4 positive (high) MVs? How? Are the OLFM4 reduced MVs preferentially taken up? This whole Fig., in the current form, raises many more questions about the rigor of the data acquisition than it adds to the scientific knowledge gain.

As mentioned above, we intended to assess OLFM4-MV function by treating HGC-27cells with OLFM4-MVs or OLFM4 reduced MVs in parallel; indeed, we used HEK293T as MV producer cells in the original manuscript. To better address this issue, we generated MVs from AGS-EBV (expressing high level of OLFM4 protein) cells transduced with shCtrl- and shOLFM4- lentivirus. Then we used these MVs to treat AGS/HGC-27 cells with endogenous OLFM4 knocked down (**Fig. R25a**). We confirmed the knockdown efficiency of OLFM4 in AGS-EBV cells and the derived MVs (**Fig. R25b**). Subsequent colony formation assays showed that OLFM4 reduced MVs showed less effect to promote colony formation when compared to OLFM4-MVs (**Fig. R25c-d**). Consistently, OLFM4 reduced MVs was less able to promote YAP target genes (CTGF and CYR61) expression.

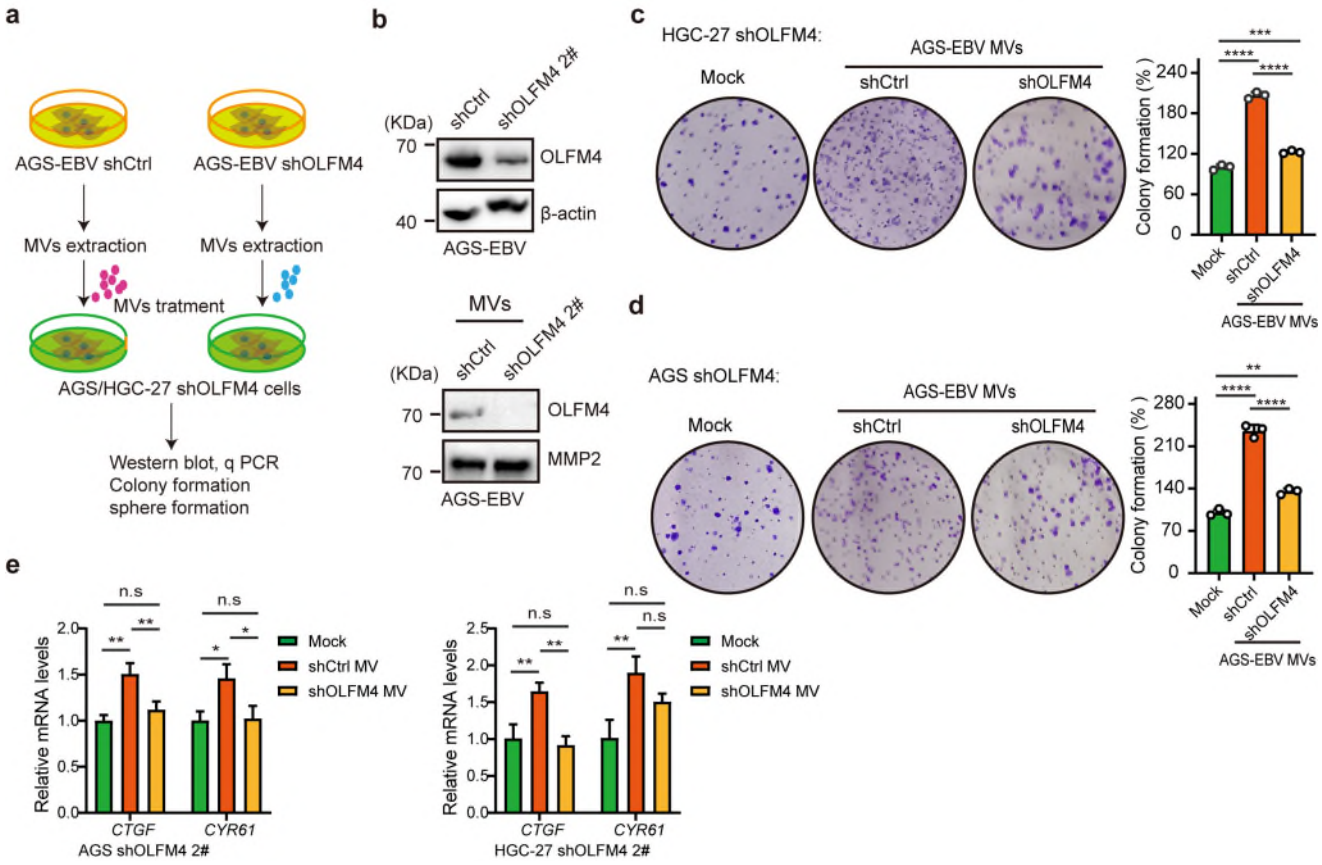


Fig. R25 MV regulation of cell growth is dependent on OLFM4 level in MVs. a, Experimental workflow. The MVs were collected from the Ctrl or OLFM4 knockdown

AGS-EBV cells. Then these MVs were used to treat AGS or HGC-27 cells in which endogenous OLFM4 was knocked down by its specific shRNA. 3×10^8 particles /ml MVs were used for each treatment. **b**, Immunoblotting showing the levels of intracellular OLFM4 in shCtrl and shOLFM4 AGS-EBV cells (upper) and the OLFM4 levels in MVs generated by these cells. **c-d**, Representative images showing colony formation and sphere formation ability of MV-treated shOLFM4 AGS and shOLFM4 HGC-27 cells. Cells were treated by 3×10^8 particles /ml MVs for 24h. Three technical replicates were performed in each group. **e**, QPCR analysis of YAP target genes (*CTGF* and *CYR61*) in shOLFM4 HGC-27 and shOLFM4 AGS cells after treatment with the indicated MVs. Cells were treated by 3×10^8 particles /ml MVs for 24h.

Based on the above, is it correct to assume that the OLFM4 overexpression derived MVs are also 293 cells are used? First of all, there is no mentioning, apart from the first confusing lines in the results text for Fig5 that 293 cells are used. Why is this omitted?

Sorry for the unclarity. Indeed, we used HEK293T as producer cells to generate MVs in order to assess the importance of OLFM4 protein in the MV function, as well as to rule out the potential contribution from other pro-proliferative factors in GC-derived MVs. We now clearly described the origin of the used MVs in the related figure legend.

Then, the authors use 293T cells-is the T-antigen included in MVs? Have evaluations been done between the different 293 derived (with or w/o OLFM overexpresison) MV on a proteomic level?

As mentioned above, HEK293T cells have been widely used as EV producer cells. Following the reviewer's comments, we performed Mass spectrometry analysis on HEK293T (with and w/o OLFM4 overexpression) MVs, and found these MVs did not contain T-antigen (**Extended Data tables 5 and 6**).

Would the mismatch between 293T MVs and hgc27 cells result in immune activation, which through the expression of OLFM4 might be altered? Especially as the authors investigate interferon responses, this is all very challenging to interpret.

As mentioned above, HEK293T cells have been widely used as extracellular vesicles (EV) producer cells due to their inherent rapid proliferation, high EV yield, and ease of genetic manipulation[20-23]. Previous studies have shown that HEK293T EVs showed low toxicity, and minimal changes in immune markers[20, 24]. Consistent with these reports, we observed similar effects on YAP signaling in HGC-27 cells receiving either HEK293T MVs or HGC-27 MVs (**Fig. R26**).

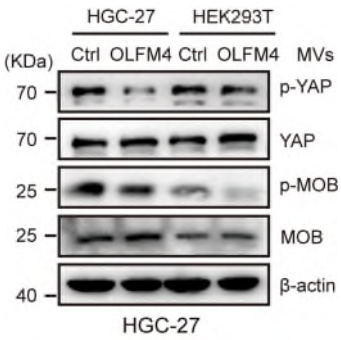


Fig R26. Comparison of HGC-27- or HEK293T- derived MVs. Immunoblotting showing the levels of p-YAP1, YAP1, p-Mob1 and Mob1 in HGC-27 cells after the indicated cell-derived MVs (3×10^8 particles/ml). All lanes were loaded with 50 μg of total protein.

Discussion: The authors might want to explore, in more detail, the role MVs or other EVs could play in cancer progression and stromal responses. Re-statement of results should be the exception and it is suggested to the authors to re-work the current discussion to focus more on the implications of the data and similar/opposing results from others rather than explaining their own data again.

Agree! Many thanks. We now re-worked our discussion to focus more on implications of the data and similar/opposite results from other studies.

"Previously, OLFM4 has been reported as a molecular marker for Intestinal stem cells and some gastrointestinal cancers[25-28]. OLFM4 has been implicated in cancer cell proliferation, cell cycle regulation, and cell adhesion and metastasis of some malignancies, especially in gastrointestinal cancers[25, 26]. It has been reported that higher expression of OLFM4 is associated with severity of disease for many viral and bacterial infections[29]. In this study, we showed that EBV and VSV but not HSV-1 can led to significant upregulation of OLFM4, suggesting a virus type-specific effect. This also explains in part why the expression of OLFM4 is more prevalent in EBVaGCs but sometimes can be detected in EBVnGCs — there might be other viruses in these samples.

Previous studies have shown that OLFM4 promotes GC cell migration and proliferation[30], and reduced expression of OLFM4 in GC is associated with lymph node and distant metastases and with poor prognosis[31]. Partially consistent with these observations, here we found that OLFM4 MVs act as an extracellular inhibitory ligand for the Hippo pathway — it is possible that OLFM4 MVs may also regulate GC metastasis via Hippo or Hippo-related pathways. Upstream of OLFM4 MVs, we identified OLFM4 as a direct target gene of the cGAS-STING signaling pathway. Upon EBV infection, activated cGAS-STING promotes OLFM4 expression via IRF3. Notably, we observed that EBV infection significantly also stimulated intracellular MMP7 expression and its MVs secretion. However, MMP7 MVs did seem to have any effect on the Hippo pathway and GC growth. Given the previous report that YAP signaling upregulates MMP7 expression and promote cell adhesion and migration[10-12], we speculate that OLFM4 MVs activate YAP signaling, which may in turn stimulate MMP7 expression and secretion."

Minor point: The sentence line 155-157 does not make sense

We now corrected the description. Many thanks!

"In keeping with these results, co-culture of AGS cells with Akata EBV+ cells derived from a Burkitt lymphoma cell line stably, also dose-dependently induced OLFM4 expression (**Extended Data Fig. 1a**)."

Reviewer #4 (Remarks to the Author):

In this study, Wen et al. proteome profiling of microvesicles (MVs) in Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC). They found that OLFM4 is a critical protein induced by EBV infection and secreted via MVs, and promotes gastric tumorigenesis through Hippo-YAP signaling. OLFM4 was a downstream target gene of cGAS-STING pathway that was activated upon EBV infection, and **MV-carried OLFM4 was an extracellular inhibitory ligand for the Hippo pathway**. They found that viral infection coupled with extracellular vesicles plays a role in control of Hippo signaling, highlighting OLFM4-Hippo axis as a potential therapeutic target for EBV associated cancers.

General comments:

Contribution of MV-carried protein in controlling Hippo signaling and tumorigenesis is potentially interesting especially in the field of virus associated tumor, although essence of this manuscript including importance of extracellular vesicles, involvement of cGAS-STING pathway in viral infection, contribution of OLFM4 in gastrointestinal cancer, does not seem so novel and may not give strong impact. The comments below are the concerns to be addressed.

We thank the reviewer for the inspiring comments. We now discussed the key findings and short points in the "limitation of this study" of the revised manuscript.

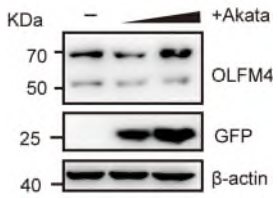
Specific comments:

1. EBV is infected to HGC-27, and RNA-seq was conducted (in Fig. 3). Although infection rate of EBV in gastric cells is generally so low, but did the authors select infected cells by drugs?

We did not select infected cells by drugs.

Materials and methods showed that they used Akata cells for infection experiments, but it is not clear whether EBV in Akata cells is recombinant EBV or not.

Following the reviewer's comment, we now added the detailed information for Akata cells used in the study. The Akata cells we used to produce recombinant EBV-EGFP virus. We detected GFP expression in the infected cells (**Extended data Fig.1a, original manuscript**).



Extended data Fig.1a, original manuscript OLFM4 was induced by virus infection. Western blot to detect OLFM4 expression in AGS cells that were infected by Akata cells-derived EBV. The EBV-positive Akata cells (1×10^5, 1×10^6) containing a GFP tag in the viral genome were co-cultured with AGS cells for 24 h. Efficiency of EBV infection was identified by

GFP expression in infected AGS cells. All lanes were loaded with 50 μg of total protein.

RNA was extracted at 24 h after infection (Fig. 3a,b), but it is not clear whether this is the effect of EBV "infection" or exposure to foreign substances. Similarly, elevation of OLFM4 was shown (Fig 3d) under extraction of proteins at 48 h, but it is not clear whether this is the effect of EBV "infection" or exposure to foreign substances.

As mentioned above, we did not select infected cells by drugs. The recombinant EBV was generated by Akata cells according to the protocol described by kassis, jareer et. al[32]. Then the EBV was used to infect AGS cells. Actually there was no other "foreign substances" except EBV because the same supernatant/solvent (without EBV) was used as a control for the assay.

Infection rate might be shown, e.g. by EBER in situ hybridization, copy number of EBV genome in cells might be shown, e.g. by qPCR, and EBV-infected cells can be selected, by utilizing drug-resistance.

We used a standard procedure for EBV infection *in vitro*[32]. In Akata cells, production of progeny viruses can be induced by cross-linking surface immunoglobulin G (IgG) molecules using anti-IgG antibodies[32]. Therefore, large quantities of pure recombinant viruses can readily be produced by anti-IgG antibody treatment[33]. EBV infection was confirmed by EBNA-1 western blot (**Fig. R27a,b**). Moreover, we also performed immunofluorescent assay to detect the EBV infection rate and observed ~20% EBNA-1-positive cells in both HGC-27 and GES-1 cells (**Fig. R27c-e**), confirming the successful EBV infection. We did not select the EBV-infected cells by drug because we want to study the initial change of the cells when EBV was infected, otherwise we'll use AGS-EBV cell line. We now incorporated these results into the revised manuscript.

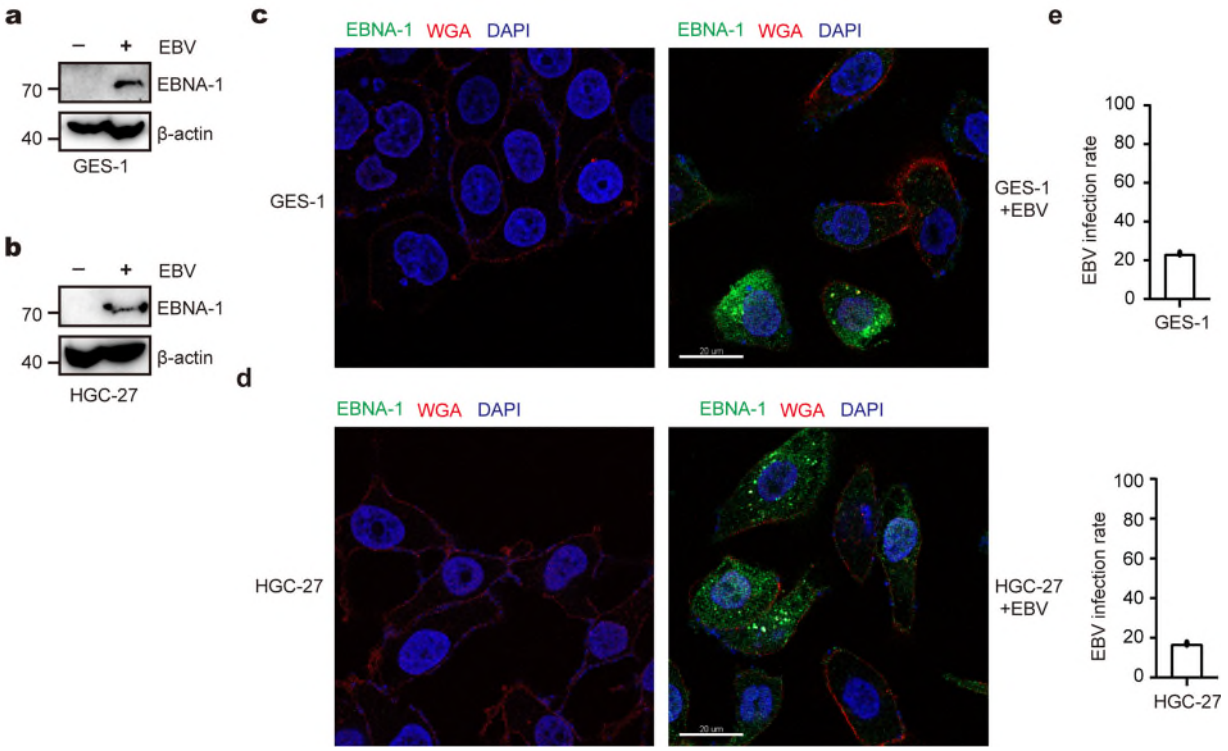


Fig. R27 Efficiency of EBV infection. a, Immunoblot detection of EBNA-1 in EBV-infected GES-1 cells. **b,** Immunoblot detection of EBNA-1 in EBV-infected HGC-27 cells. **c,** Immunofluorescent assay showing EBNA-1 (green) and WGA (plasma membrane, red) staining in EBV-infected GES-1 cells. Scale bars, 20 μm. **d,** Immunofluorescent assay showing EBNA-1(green) and WGA (plasma membrane, red) staining in EBV-infected HGC-27 cells. Scale bars, 20 μm. **e,** Bar graph showing EBV infection rate in GES-1 and HGC-27. Fifty cells were quantified for EBNA-1 staining.

2. MV treatment on GC cell line was performed using HGC-27 (Fig. 5a) without replicate. This experiment should be done using multiple cell lines, by adding at lease another cell line.

Agree! Regarding Fig.5a, we performed the experiment twice, each with three biological replicates. Representative images and quantification from one experiment were shown. We now added the related description in figure legends.

Following the reviewer's comments, we performed the related experiments in another cell line. We used AGS cells to re-perform colony formation assay and sphere formation assay (**Fig. R28**). Similar to the observations in HGC-27 cells (**Fig.5a, original manuscript**), OLFM4-overexpressing MVs dose-dependently increased the colony formation (**Fig. R28a**) and sphere formation of AGS cells (**Fig. R28b**). We now incorporated these data in the revised manuscript.

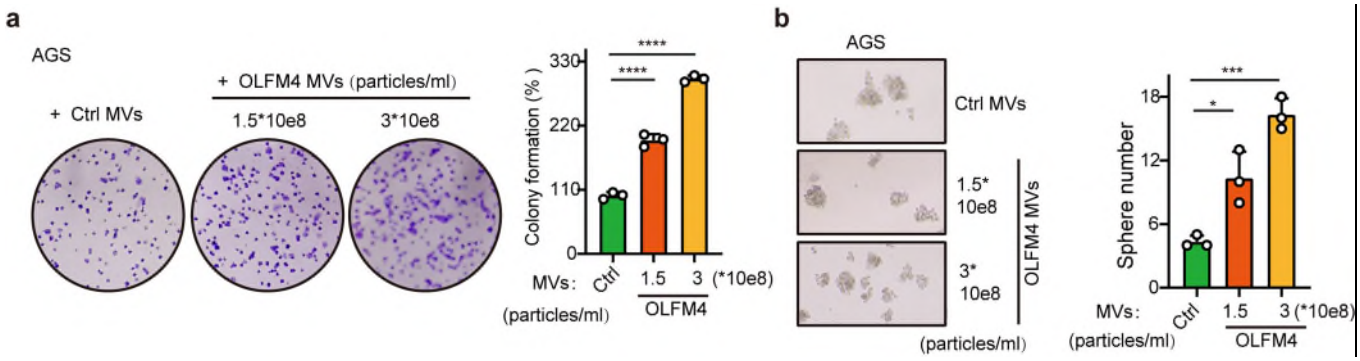


Fig. R28 OLFM4-overexpressing MVs promotes colony formation and sphere formation of AGS cells. a, Representative images (left) and quantification (right) showing colony formation ability of AGS cells treated with OLFM4-overexpression MVs isolated from HEK293T cells. 2000 cells were seeded in each well and cultured for 7 days. **b,** Representative images (left) and quantification (right) showing sphere formation ability of AGS cells treated with OLFM4-overexpression MVs. Assays were carried out with 3 biological replicates.

3. shRNA knockdown on MV-treated GC cell line was performed (Fig. 5b), but this was also conducted using HGC-27 only. The authors should do this experiment using multiple cell lines, by adding at lease another cell line.

Following this reviewer's suggestion, we also used AGS cells to re-perform colony formation assay and sphere formation assay (**Fig. R28**). The results showed that OLFM4 MVs promote cell proliferation of HGC-27 and AGS cell lines. We now incorporated these results into the revised manuscript. The experiment design in Fig. 5b of previous version of manuscript is unsuitable as mentioned by Reviewer 3 and Reviewer 4, so we replaced the experiment by using a EBV-positive AGS-EBV model according to the reviewer's suggestion (**Fig. R29**). We knocked down OLFM4 in AGS-EBV cells and then purified MVs from them to treat HGC-27 shOLFM4 or AGS shOLFM4 cell lines (**Fig. R29a,b,c**). Compared to the shCtrl MVs, OLFM4-knockdown MVs isolated from EBV-infected cells showed reduced ability to promote colony formation (**Fig. R29d,e**) and sphere formation (**Fig. R29f,g**) of HGC-27 and AGS cells, emphasizing a functional role for OLFM4 in the MVs. Except that, we also knocked down OLFM4 in HGC-27 and AGS cells (**Fig. R30a,b**), and found that knock down OLFM4 significantly suppressed GC cell colony formation (**Fig. R30c,d**) and sphere formation (**Fig. R30e,f**). We now incorporated these data in the revised manuscript.

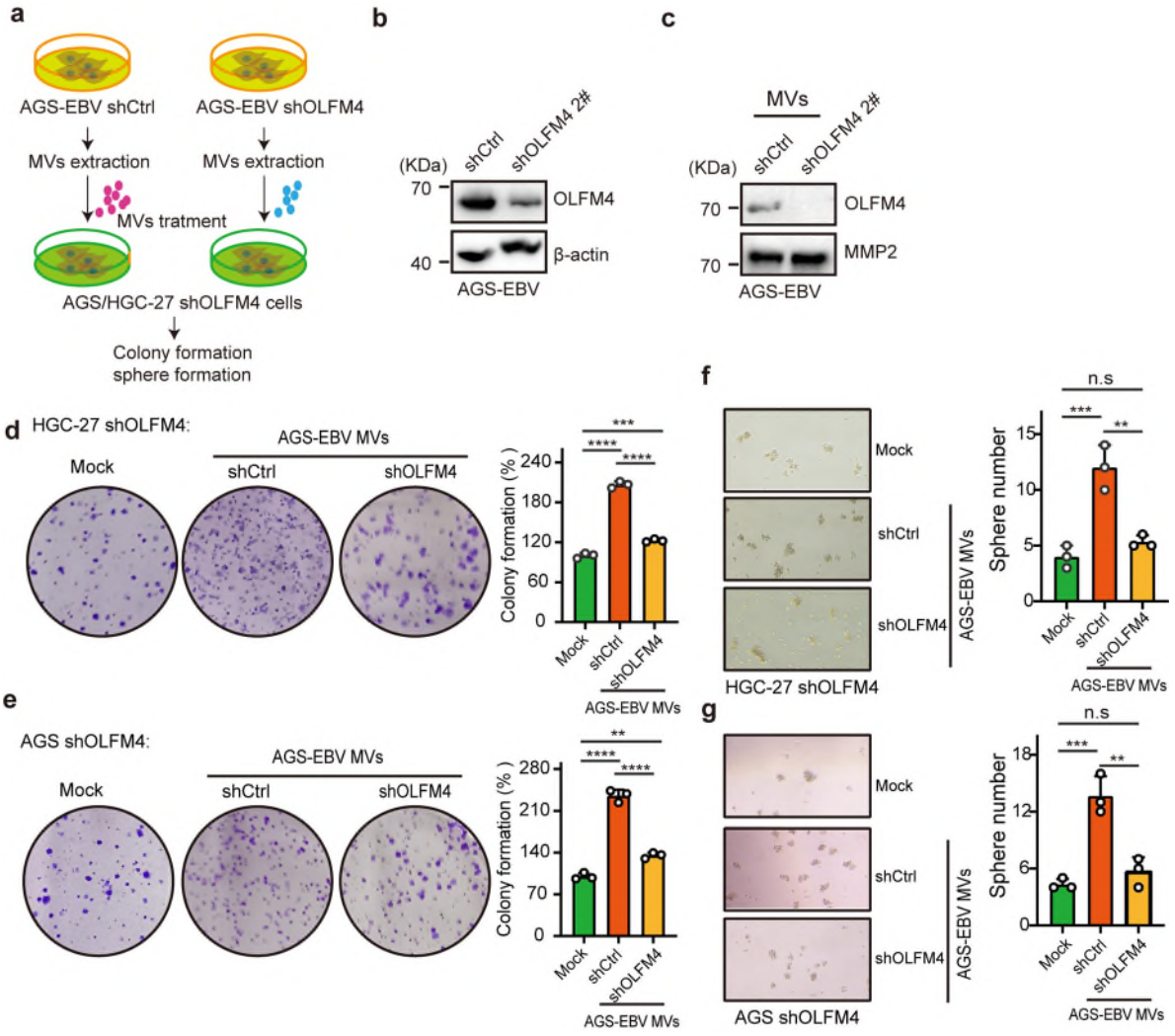


Fig. R29 Functional assessment of OLFM4 in MVs derived from EBV infected cells. **a,** an experimental workflow. **b,** Immunoblotting and QPCR assay showing the levels of intracellular OLFM4 in OLFM4-knockdown AGS-EBV cells. Scramble shRNA was used as a control. **c,** Immunoblotting showing the levels of OLFM4 in MVs of OLFM4-knockdown AGS-EBV cells. (**d-e**) Representative image showing colony formation of HGC-27 (**d**) or AGS (**e**) cells treated with MVs derived from shOLFM4 AGS-EBV cells. The colonies were grown for 1 week and stained by crystal violet. The number of cell colonies was counted by ImageJ software. Relative percentage of clone number +/- s.e.m is shown. (**f-g**). Sphere formation assay for HGC-27 (**f**) and AGS (**g**) cells treated with MVs derived from shOLFM4 AGS-EBV cells. Representative images and barplots of sphere number quantification are shown.

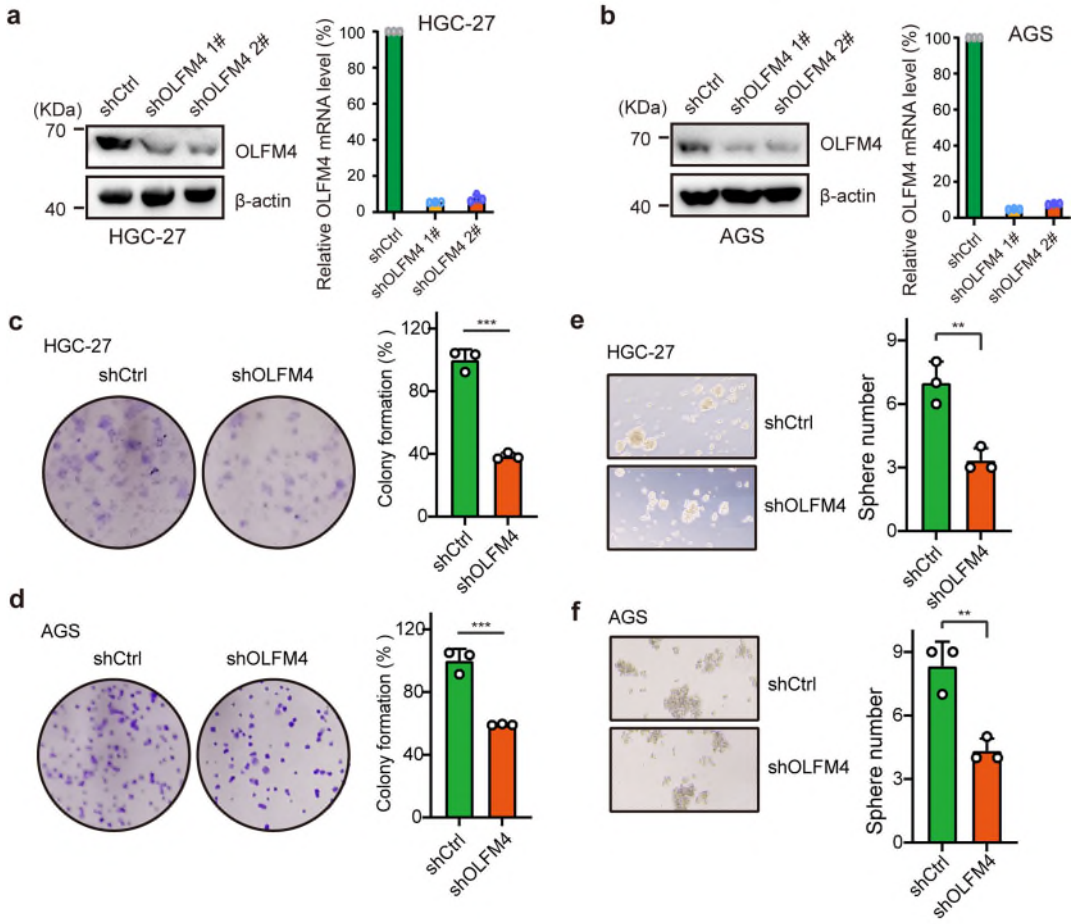


Fig. R30 OLFM4 knock down inhibits GC cell growth. (**a-b**), Detection of OLFM4 knockdown efficiency in HGC-27 (**a**) and AGS (**b**) cells. OLFM4 was knocked down by shRNA in HGC-27 or AGS cells, and the knockdown efficiency was confirmed by western blot and qPCR. Two shOLFM4 clones were used, the scramble shRNA was used as control. shOLFM4 2# clones were used for colony formation and sphere formation assays. (**c-d**) Representative image showing colony formation of OLFM4 knockdown- HGC-27 (**c**) or AGS (**d**) cells. Mediums were replaced every other day. The colonies were grown for 1 week and stained by crystal violet. The number of cell colonies was counted by ImageJ software. Relative percent of clone number +/- s.e.m is shown. (**e-f**). Sphere formation assay was performed with OLFM4 knockdown HGC-27 (**e**) and AGS (**f**) cells. Representative images and bar plots of sphere number quantification are shown.

4. shRNA experiment is also very confusing and unclear. Fig. 5b legend shows "30 μg HGC-27 shCtrl or shOLFM4 cell lines derived MVs were used for the treatment", but were shCtrl and shOLFM4 treated with HGC-27? If so, why MV-treated HGC-27 must be treated by shOLFM4? Or, was shRNA treated with HEK293T? If so, the target of shOLFM4 would seem to be overexpressed OLFM4 in HEK293T cells, but such experiment is usually done overexpressing function-lost OLFM4, not shRNA knockdown.

Sorry for the unclarity. Yes, we used HEK293T to generate MVs in order to exclude

other potential pro-proliferative factors in GC-derived MVs (**Fig. R31**). For example, EVs have been shown to carry a number of herpesvirus-associated proteins, including LMP1, EGFR and PI3K, from the host cells to the recipient cells to accelerate cell growth[13-15]. By knockdown OLFM4 in HEK293T cells, we intended to assess the importance of OLFM4 in MV regulation of AGS/HGC-27 cells.

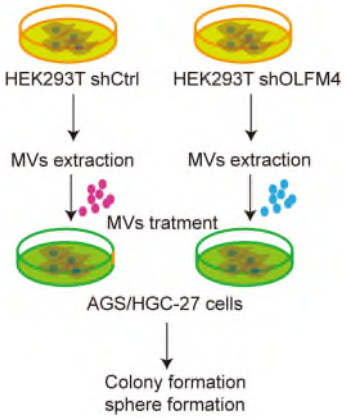


Fig. R31 Experimental workflow. MVs were collected from the OLFM4 knockdown HEK293T cells and subsequently used for treatment of AGS/HGC-27 cells.

We agree with the reviewer that overexpression of a function-lost OLFM4 instead of knockdown would be better to strengthen our study. To this end and to distinguish between intracellular OLFM4 and secretary OLFM4, we constructed a signal peptide deletion mutant of OLFM4, OLFM4 (DelSP), which lacks the N-terminal 1-20 aa and therefore could not be secreted into MVs (**Fig. R13a**). Of note, we found that these MVs isolated from the OLFM4 (DelSP)-transfected cells failed to activate YAP signaling (**Fig. R13bc**) and to promote colony formation (**Fig. R13d,e**) in HGC-27 cells. Except that, we knocked out OLFM4 in HGC-27 cells, then restore its intracellular function by transfect with DelSP OLFM4 (**Fig. R32 a**). Quantitative PCR, cell proliferation, and cell viability assays showed that HEK293T derived MVs with OLFM4 significantly promoted the cell proliferation, but the MVs generated by HEK293T expressing DelSP OLFM4 which not secrete OLFM4 not (**Fig. R32 b-d**). These results clearly indicate that EBV-infected GC cells secrete OLFM4-containing MVs to stimulate overgrowth of the neighboring cells that are not infected with EBV (**Fig. R13f**).

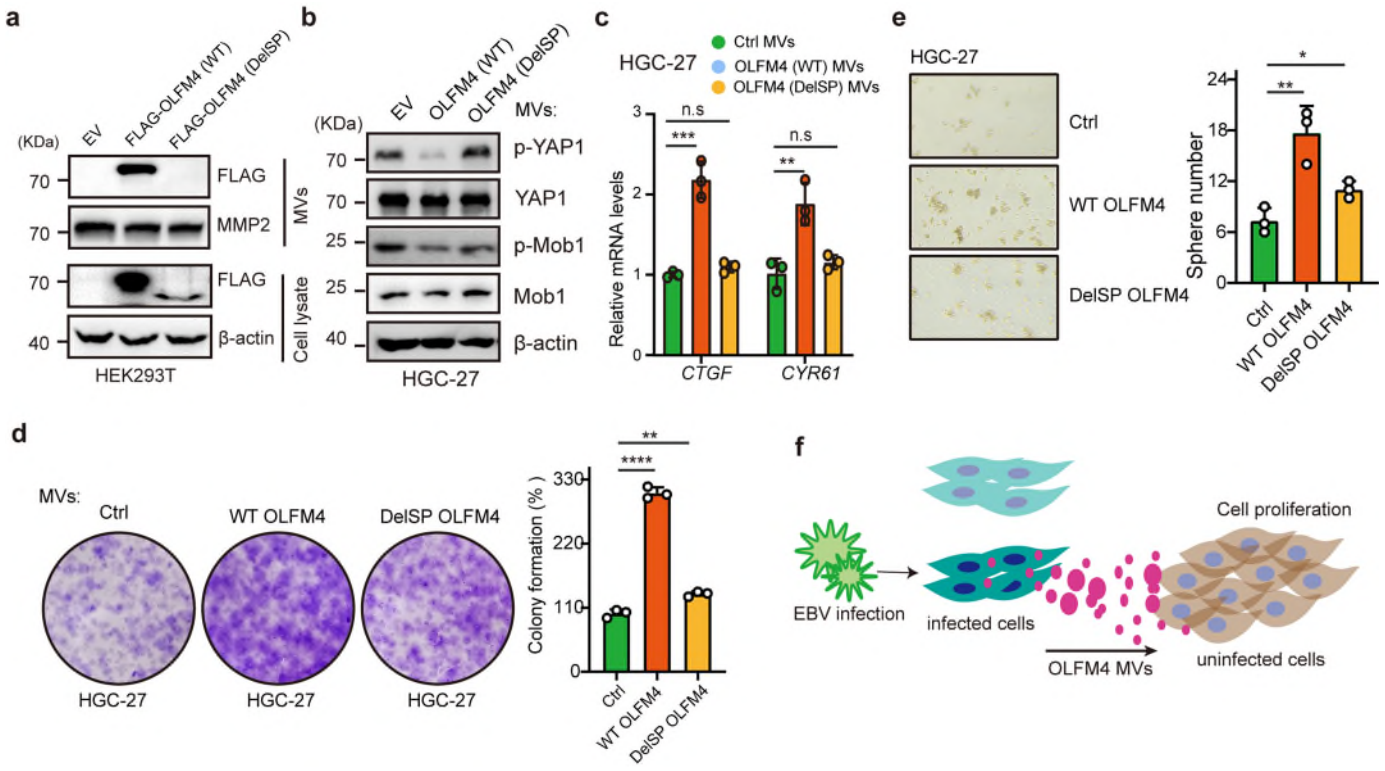


Fig. R13 Comparison of wildtype and a secretion-disabled mutant OLFM4. **a**, Immunoblotting showing the levels of WT or mutant OLFM4 in OLFM4 overexpressing MVs in HEK293T cells. WT, wildtype. FLAG tag was inserted after signal peptide of OLFM4. **b,** Immunoblotting showing OLFM4 expression in tumor tissues and their MVs of GC patients. **c**, Immunoblotting of p-Mob1, Mob1, p-YAP1 and YAP1 in HGC-27 cells treated with empty vector (ctrl)-, wildtype OLFM4- or its deletion mutant MVs. **d**, QPCR analysis of YAP target genes (*CTGF* and *CYR61*) in HGC-27 cells treated with the indicated MVs. **e**, Colony formation of HGC-27 cells treated with the indicated MVs. **f**, Diagram. EBV-infected GC cells secrete OLFM4-containing MVs to stimulate overgrowth of the neighbouring cells.

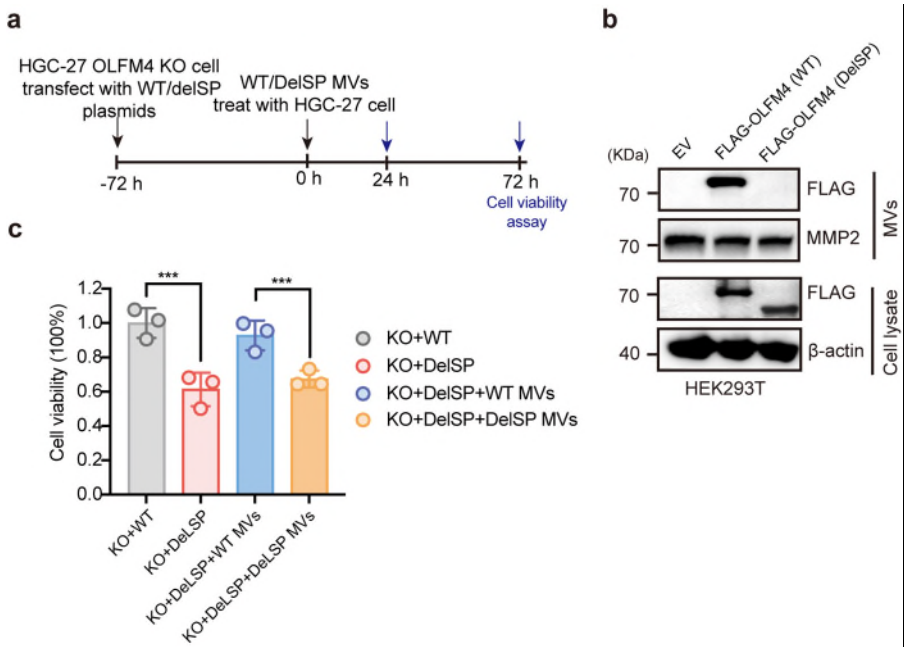


Fig. R32 Secrete OLFM4 promote cell growth and YAP activity. (**a-b**). An experimental work flow. OLFM4 was knocked out in HGC-27 cells, and these cells were then transfected with wildtype (WT) or DelSP OLFM4. The HGC-27 KO cells reconstituted with wildtype or DelSP OLFM4 were treated with MVs generated from HEK293T cells overexpressing wildtype or DelSP OLFM4. (**b**) Immunoblotting to detect OLFM4 expression in HEK293T cells and generated MVs. WT and DelSP OLFM4 were cloned in pcDNA3.1(+) vector with a FLAG tag after signal peptide. HEK293T cells were transfected with the WT or DelSP OLFM4, after 48 h, the supernatant was collected for MV extraction. For cells lysate, each lane loaded with 50 μg of total protein. For MVs, each lane loaded 2 x 10^8 MV particles. (**c**) Plots showed the secreted OLFM4 significantly promoted the cell viability. Cells were seeded into 96-well plates at a density of 1000 per well overnight to have them become attached to the wells, and then treated with HEK293T-delivered MVs (3×10^8 particles/ml). The cells were incubated for 3 d, and then detected by a ATPbased CellTiter-LumiTM Plus kit. Bar graphs show mean +/− s.e.m. ***$P < 0.001$, n.s, not significant.

5. Perturbation experiment on OLFM4, such as shOLFM4, should additionally done on EBV-positive GC cell lines, and EBV-infection model.

Following the reviewer's comment, we knocked down OLFM4 in AGS-EBV cells and

then purified MVs from them to treat HGC-27 shOLFM4 or AGS shOLFM4 cell lines (**Fig. R29a,b**). Compared to the shCtrl MVs, OLFM4-knockdown MVs isolated from EBV-infected cells showed reduced ability to promote colony formation (**Fig. R29b,c**) and sphere formation (**Fig. R29d,e**) of HGC-27 and AGS cells, emphasizing a functional role for OLFM4 in the MVs.

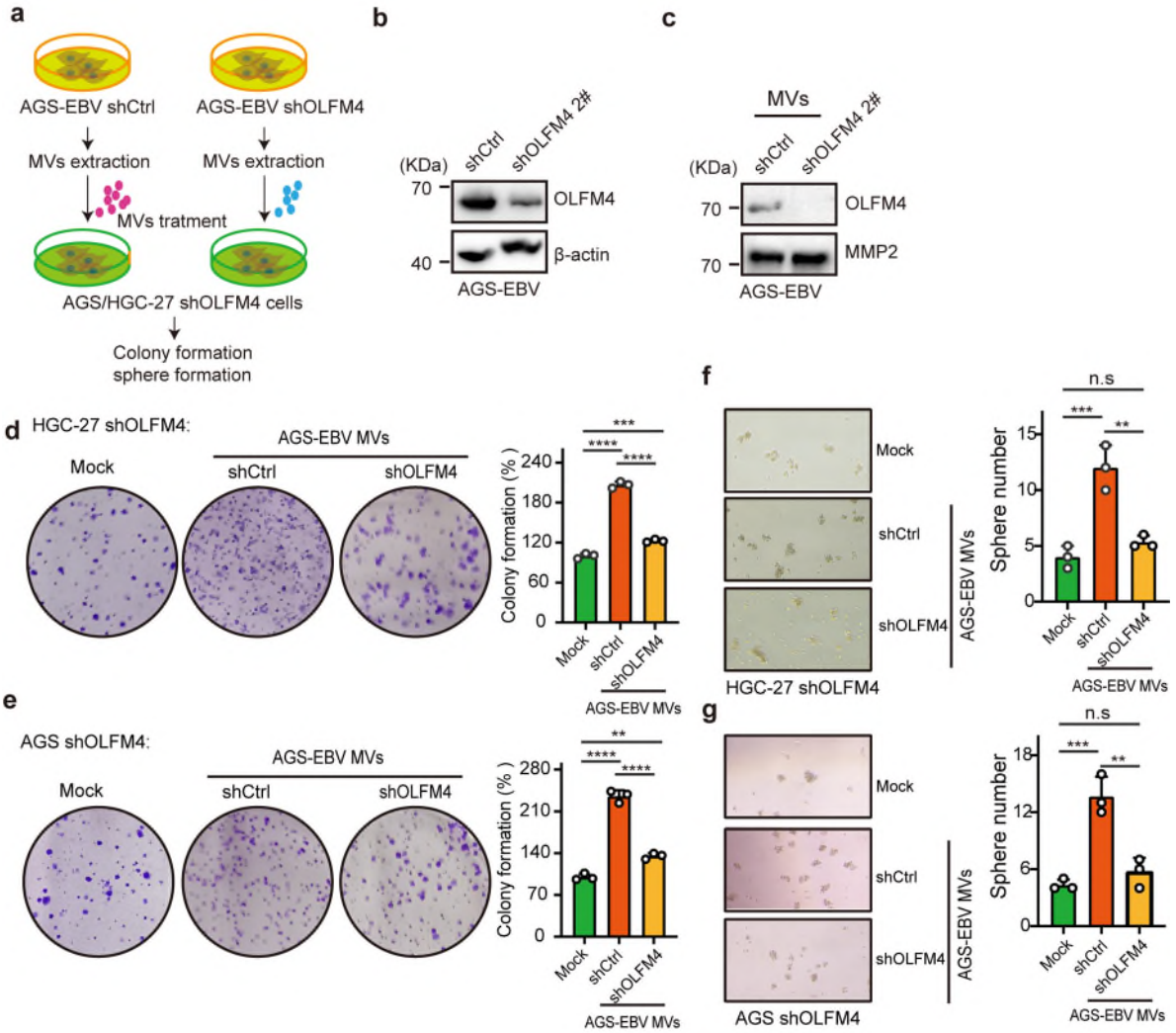


Fig. R29 Functional assessment of OLFM4 in MVs derived from EBV infected cells. **a,** an experimental workflow. **b,** Immunoblotting and QPCR assay showing the levels of intracellular OLFM4 in OLFM4-knockdown AGS-EBV cells. Scramble shRNA was used as a control. **c,** Immunoblotting showing the levels of OLFM4 in MVs of OLFM4-knockdown AGS-EBV cells. (**d-e**) Representative image showing colony formation of HGC-27 (**d**) or AGS (**e**) cells treated with MVs derived from shOLFM4 AGS-EBV cells. The colonies were grown for 1 week and stained by crystal violet. The number of cell colonies was counted by ImageJ software. Relative percentage of clone number +/- s.e.m is shown. (**f-g**). Sphere formation assay for HGC-27 (**f**) and AGS (**g**) cells treated with MVs derived from shOLFM4 AGS-EBV cells. Representative images and barplots of sphere number

quantification are shown. Bar graphs show mean +/− s.e.m. **P < 0.01, ***P < 0.001, ****P < 0.0001, n.s, not significant.

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A Point-by-point Responses to the Reviewers' Comments

Reviewer #1 (Remarks to the Author):

Authors have successfully addressed the reviewer's comments. The manuscript is ready to be accepted for publication.

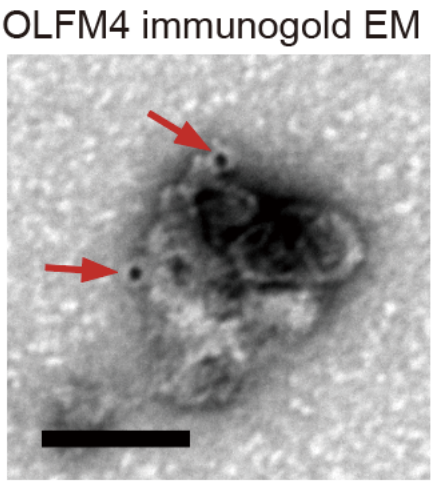
We thank the reviewer for the constructive comments during the revision of our manuscript.

Reviewer #2 (Remarks to the Author):

The revised manuscript has been much improved and addresses some of my concerns. The authors propose a model in which cancer produces OLFM4-carried MVs that bind to the extracellular domain of FAT1 on recipient cells to inhibit Hippo signaling. However, it remains unclear whether OLFM4 is embedded inside the vesicle or anchored on its surface. OLFM4 can only exert its functional activity if it is localized on the vesicle membrane surface.

Alternatively, OLFM4-carried MVs might be engulfed by the recipient cell, and released intracellular OLFM4 can also inhibit Hippo signaling, as demonstrated by the authors' new data. How does intracellular OLFM4 inhibit Hippo signaling? It appears to do so through mechanisms distinct from those of extracellular OLFM4. The authors should provide clear evidence demonstrating whether the inhibition of Hippo signaling in the recipient cells is caused by extracellular or intracellular OLFM4-carried MVs.

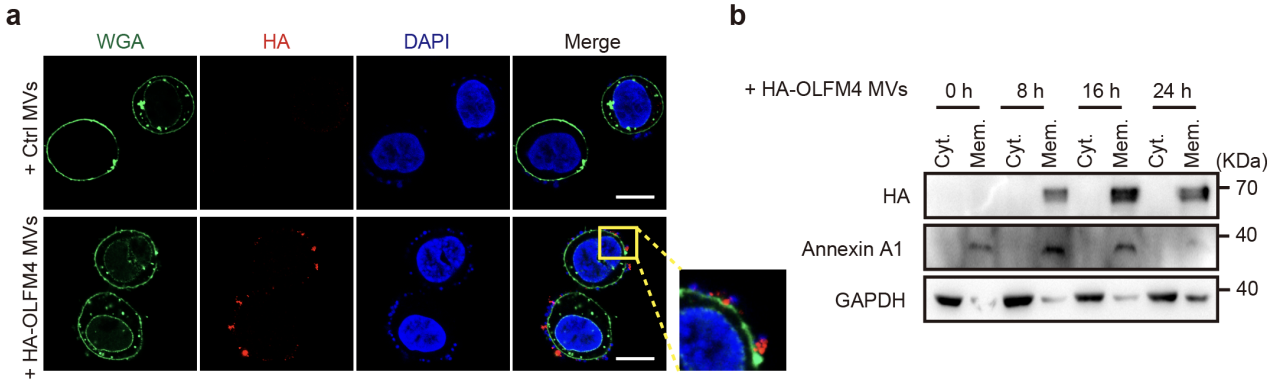
Regarding the reviewer's concern of "whether OLFM4 is embedded inside the vesicle or anchored to its surface", we applied immunogold-labeling transmission electron microscopy and showed that OLFM4 is most likely anchored on the surface of MVs (**original Fig.2a**). Specifically, we placed MVs on formvar carbon-coated copper grids and then incubated them with the goat anti-rabbit secondary antibody conjugated to 10 nm gold particles. Therefore, only OLFM4 protein on the MV surface can be labelled; whereas those embedded inside the vesicle could not be labelled.



Original Fig. 2a

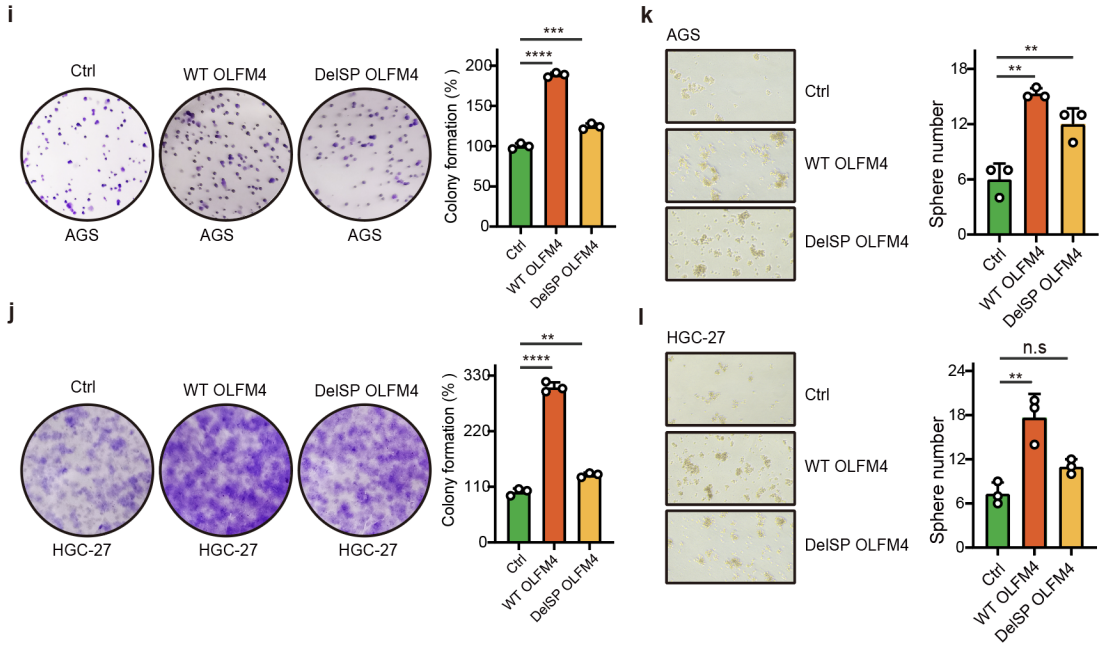
Moreover, our immunofluorescence and immunoblotting results revealed that MV-delivered OLFM4 was localized on the plasma membrane of the recipient cells (**original Fig.7a,b**). Notably, there was no significant OLFM4 signal in the cytoplasm of recipient cells (**original Fig.7a,b**), further indicating that OLFM4

anchors on MV surface and transferred onto the plasma surface of recipient cells during the fusion of MVs to the recipient cells.



Original Fig. 7a,b

Meanwhile, we observed that overexpression of a mutant variant of OLFM4 (DelSP), which lacks the N-terminal signal peptide of OLFM4 and therefore disabled for MV-mediated secretion, was still able to promote cancer cell proliferation (**Original Extended Data Fig. 6i-l**). However, the pro-proliferative effect of this mutant variant was reduced when compared to WT OLFM4 (**Original Extended Data Fig. 6i-l**). Together with the above results, these observations further indicate that MV-carried OLFM4 regulates recipient cells in an extracellular manner.



Original Extended Data Fig. 6i~6l

In addition, we also performed immunofluorescent assay to investigate the colocalization of HA-OLFM4 with Flag-tagged WT or truncated variants of FAT1 (**Fig. R1**). We found a significant colocalization signal for HA-OLFM4 and WT Flag-FAT1 (**Fig. R1**). By contrast, such colocalization signal was undetectable in the case of truncated variants of FAT1, including residues 3790-4588 lacking the N-terminal cadherin repeats #28-33 or residues 4023-4588 lacking the cadherin repeats #28-33 and transmembrane sequence (**Fig. R1a**). Co-immunoprecipitation assay confirmed that intracellular region (residues 3790-4588) of FAT1 can't interact with OLFM4 (Del SP) (**Fig. R2a**). These observations further suggest that MV-carried OLFM4 interacts with the extracellular region of FAT1 and do so only when FAT1 is localized on the plasma surface.

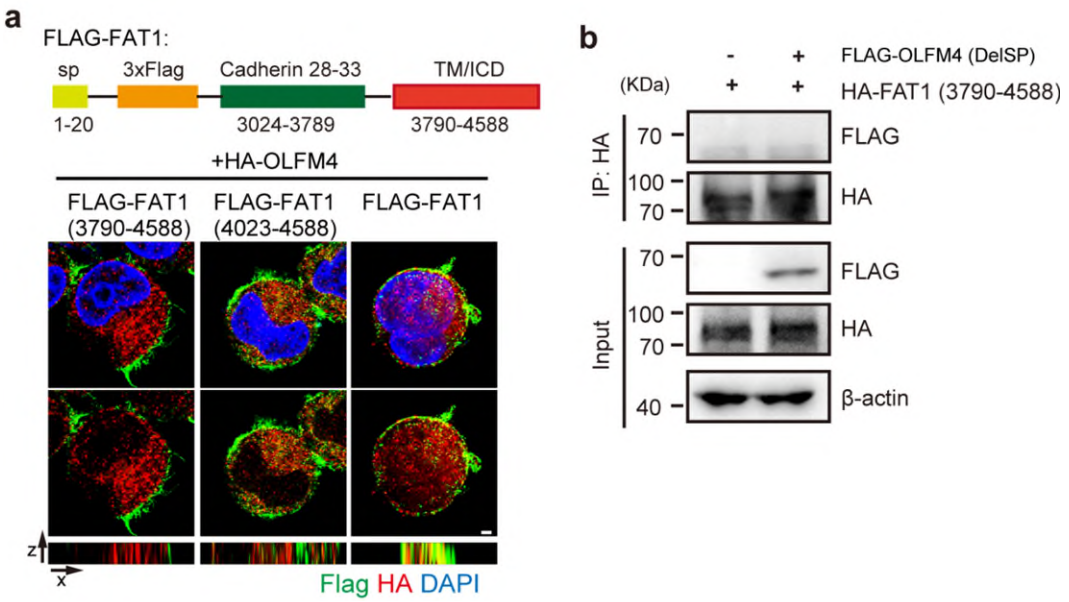


Fig. R1 OLFM4 interacts with the extracellular region of FAT1. a, Immunofluorescent assay showing the colocalization of HA-OLFM4 and FLAG-FAT1 and its truncations. Upper, construction of FLAG tagged FAT1 with cadherins #28-33. Lower, immunofluorescent images. **b**, Co-IP assay showing the interaction of HA-FAT1(3790-4588) with FLAG-OLFM4 (DelSP).

Collectively, our findings strongly indicate that MV-carried OLFM4 mainly anchors on the MV surface and transferred onto the plasma surface of recipient cells during the fusion of MVs to the recipient cells, and that these surface-anchored OLFM4 only interacts with membrane FAT1 but not cytosol FAT1 to inhibit Hippo signaling in recipient cells.

Another minor question is why the cGAS-STING pathway is suppressed by HSV1 infection. It is widely known that HSV1 can activate cGAS-STING signaling.

Agree! We speculated that this might be due to the quality of the HSV-1 used in our experiment. To address this issue, we purchased new HSV-1 (KOS strain) and re-performed the experiment. We found that HSV-1 treatment dose-dependently induced the upregulation of *IFNB* transcription in HGC-27 cells (**Fig. R2a**). At the same time, we observed that HSV-1 infection promotes OLFM4 expression in a dose-dependent manner (**Fig. R2b**). Similar observation was obtained by immunoblotting (**Fig. R2c**). We now incorporated these results into the revised manuscript with discussion of possible effect of viruses other than EBV.

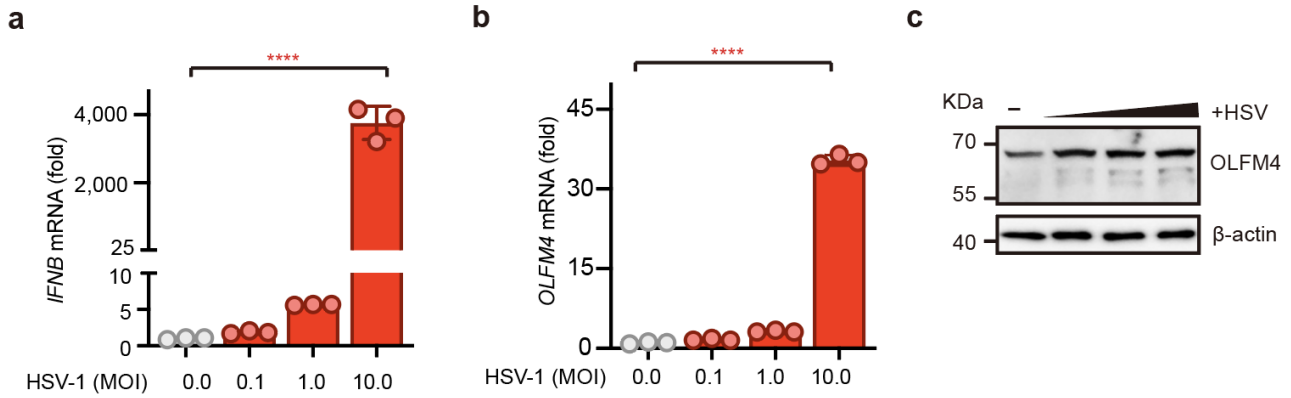


Fig. R2 HSV-1 treatment induces OLFM4 upregulation. a, mRNA levels of *IFNB* in HGC-27 cells after infection with HSV-1 for 48 h. **b**, mRNA levels of *OLFM4* in HSV-infected cells. **c**, Protein levels of OLFM4 in HGC-27 cells infected with HSV-1 (MOI 0, 0.1, 1, 10) for 48 h.

Reviewer #3 (Remarks to the Author):

The authors of the manuscript "Olfactomedin 4 is a direct target of the cGAS-STING pathway and acts as an extracellular inhibitor for the Hippo signaling in EBV-associated gastric cancer" have made a tremendous effort addressing the various comments and concerns of the reviewers, including mine.

We thank the reviewer for the encouraging comments.

While most of the points raised in my previous assessment are addressed, I want to reiterate that the use of 293T derived EVs is a concern. Referring to a couple of publications by others as statement that this was used previously and therefore is 'fine' is questionable. However, instead of instigating a complete immunological evaluation, which would be utterly out-of-line for the scope of the manuscript and the already very comprehensive complexity, would the authors be able to amend the (much improved) discussion to touch on the limitations and implications of using EVs (even low speed MVs) from virus infected cells (referring to my concerns about EBNA/EBV) and 293T donor cells? It is certainly something we, as a field, need to keep in mind, and if this important gastric cancer manuscript provides some thoughts as part of the discussion, this would be highly relevant for future work.

Agree! Following the reviewer's suggestion, we now discussed the limitation of our study and highlighted the implications of using EVs or MVs from EBV-infected cells and 293T donor cells.

"**Limitations of this study**_HEK293T cells have been widely used as EV producer cells due to their inherent rapid proliferation, high EV yield, and ease of genetic manipulation[1-4]. The stem cells or metabolic active cells are thought to internalize significantly more HEK293T EVs than terminally differentiated cells[1]. Theoretically, as most EVs under evaluation are derived from human cells, they may elicit immune responses in mice. However, some studies have assessed the immune response and toxicity in mice, and found that HEK293T EVs showed low toxicity, and minimal changes in immune markers[1,5]. Our study demonstrated a new mechanism though which viral infection is coupled via MVs with intercellular control of the Hippo signaling. Although our mass spectrometry analysis on HEK293T (with and without OLFM4 overexpression) MVs, and found these MVs did not contain T-antigen (**Extended Data tables 5 and 6**), we do not fully elucidate whether MVs from EBV-infected cell or HEK293T cell may elicit immune response in recipient cells. Further investigations are warranted to address this issue and better define the toxicity and immune response of these used MVs."

Reviewer #4 (Remarks to the Author):

Wen et al. revised their manuscript on microvesicles (MVs) in Epstein-Barr virus (EBV)-associated gastric cancer (EBVaGC). They found that viral infection coupled with extracellular vesicles plays a role in control of Hippo signaling, highlighting OLFM4-Hippo axis as a potential therapeutic target for EBV associated cancers. This Reviewer #4 raised several concerns for the originally submitted version, considering some unclear, confusing, or weak parts of the manuscript. The concerns have been addressed by the authors in this revision, but the authors are still encouraged to do additional experiments to polish their data.

We appreciate the reviewer's the encouraging and constructive comments.

Specific comment 1. The reviewer understands that recombinant EBV-EGFP virus was infected, and that GFP and EBNA-1 were confirmed to be expressed after the Akata treatment. Although infection rate of EBV in GES-1 and HGC-27 was not so low, approximately 20%, EBV-infected cells were not selected and the data was therefore not very clear. It should not be very difficult, and the authors are encouraged to sort EGFP-positive cells to select (or enrich) EBV-infected cells, and add some validation experiments using selected cells.

Nice suggestion! Following the reviewer's comments, we isolated EBV-EGFP-infected HGC-27 cells after Akata treatment using FACS. Similar to our immunofluorescent observation (**Fig. R3a**), Akata treatment induced 19.9% or 19.6% infected GFP$^+$ cells in GES-1 and HGC-27 cells, respectively (**Fig. R3b**). Surprisingly, EBV-infected cells (GFP$^+$) showed a significant increase (>20000 fold) in OLFM4 transcription when compared to non-infected cells (GFP$^-$). Moreover, OLFM4 transcription in EBV-infected HGC-27 cells was higher than that in EBV-infected GES-1 cells (**Fig. R3c**), suggesting that cancer cell is more sensitive to EBV infection.

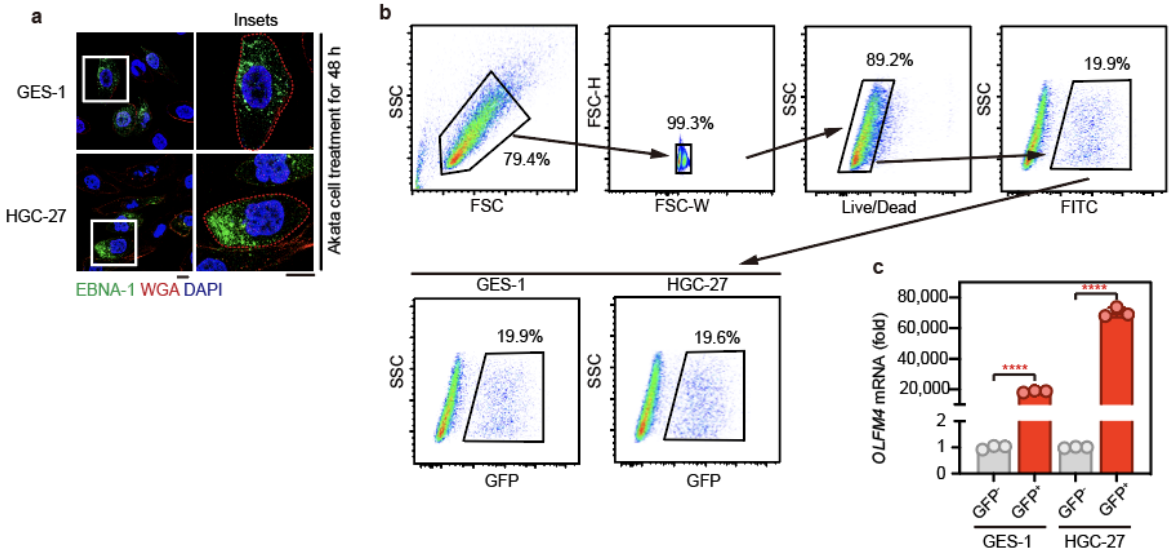


Fig. R3 EBV infection led to OLFM4 upregulation in recipient cell. a, immunofluorescent assay of GFP-positive cells after Akata treatment for 48 h. **b**, FCS showing the proportion of GFP-positive cells in GES-1 and HGC-27 cells. **b**, QPCR analysis of *OLFM4* in EBV-infected GES-1 and HGC-27 cells.

Specific comments 2-5. The authors have properly addressed all the concerns. Experiments using another cell line for MV treatment and shRNA expreiments are welcome addition. Overexpression of function-lost OLFM4 is also satisfactory, and their findings have been strengthened.

Many thanks!

Reference

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A Point-by-point Responses to the Reviewers' Comments

Reviewer #2 (Remarks to the Author):

The authors have adequately addressed my concerns.

We thank the reviewer for the constructive comments during the revision of our manuscript. Many Thanks!

Reviewer #4 (Remarks to the Author):

In the revised manuscript, the authors have properly responded to this reviewer's request that they should sort EGFP-positive cells to select EBV-infected cells, and they have added supplementary figures to show the results.

We appreciate the reviewer's the encouraging and constructive comments during the revision of our manuscript. Thanks!