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FLRT2 and FLRT3 act as repulsive guidance cues for Unc5-positive neurons

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 19 January 2011

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting, but they also indicate that significant revisions are needed in order to consider publication in the EMBO Journal. In particular they find that further data is needed to support the conclusion that FLRT proteins act as repulsive guidance cues by binding to Unc5B. The referees offer a number of comments and suggestions for how to further improve the study. Should you be able to address the criticisms of the reviewers in full, then we would consider revised manuscript. Acceptance of your paper will be dependent upon persuading the referees that you have provided a sufficient amount of new data to answer all their criticisms. I should also add that it is EMBO Journal policy to allow a single round of revision only and it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

best wishes

Editor

The EMBO Journal

REFEREE REPORTS

Referee #1 (Remarks to the Author):

Yamagishi and co-workers investigate here interactions of ligands of the FLRT falily with unc5-positive neurons. The manuscript contains a number of diverse techniques including pull downs, binding assays, in vitro assays, in situ hybridisations and in utero electroporations. They investigate the shedding of FLRT ligands from transfected cells and primary neurons in vitro, determine binding specificities, show in growth cone and stripe assay experiments repellent interactions between FLRTs and unc5-expressing cells and provide detailed analysis of migration of unc5D-positive cells in the cerebral cortex including an analysis of mice mutant for flrt2 or after electroporation-mediated overexpression of flrt2. Latter data suggest that a late-migrating sub-population of neurons is specifically affected by changes in expression of FLRT2.

Comments to figures:

Fig 3G: it is unclear which data to which data are statistically significant

Fig 3: the scatchard plots for unc5B with FLRT2 and unc5D with FLRT3 are missing.

Fig3 M-S: What is 'representative quantifications'?

Fig 4A-C: The growth cones are not visible.

Fig 4D: What is used here as a control to unc5B-ECD? What is the result when using unc5D-ECD? What is the molar ratio of unc5B to FLRT3 in these competition experiments?

Fig 4E: I don't understand the figure. Why is there an increase in growth cone collapse without expression of unc5B? Then, it appears that the unc5B siRNA has no effect on growth cone collapse rate after unc5B expression. What type of neurons is transfected?

Fig. 4F: The quantification of knock-down efficiency missing, which might be actually less than 50%?

Fig. 4G: Only one independent experiment is shown for growth cone collapse of FLRT2/3-Fc on unc5D mutant axons and needs more experiments. Is the difference in collapse rate between wt and unc5D negative axons in response to FLRT2 significant?

Fig 4H: A 'similar effectiveness' can only be demonstrated in a dose-response curve. What is the amount of unc5D-ECD used here that is the molar ratio to FLRT-2-His?

Fig. 5. Stripe assay experiments in the presence of soluble unc5B and/or unc5D are missing to demonstrate specificity. Also, it appears that for the most part the soma of hippocampal cells are repelled from green Fc stripes in control Fc-Fc assays, leading to an apparent discrepancy between picture F and data in G.
The overlay with green is not helpful as it covers most of the neurites.

Fig. 6. I wonder why in A the number of cells on Fc and FLRT2 stripes is still the same after 2 h, while the time lapse in D shows that cells move much faster, being repelled within a 2 h window twice already.

Fig. 7. FLRT2 and unc5D are expressed in different regions of the brain for the most part, I would not call this complementary as there are huge gaps between their expression domains, or at least it does not suggest some regulatory interactions between FLRT2 and unc5D positive cells.

Fig. 8. Crucially, there is an obvious discrepancy in the location of the flrt2 RNA relative to the corresponding protein (Fig 7C and 7D). The authors provide compelling evidence for shedding of the protein, however, the pattern seen in Fig. 7D is not at all what I would expect from the local

RNA pattern shown in 7C.
I would be interested to see the in situ data at E19/20 for flrt2 and unc5d.

Overall, I am not at all convinced that the data presented suggest a model proposed by the authors including a transient downregulation of the molecules investigated during migration. Actually this model should be supported by further antibody-staining for FLRT2-positive/negative cells during the migration of this particular population to the cortex though I am not sure whether antibodies are available so that this population can be identified.

Referee #2 (Remarks to the Author):

This is an interesting manuscript that presents evidence that Unc5 netrin receptor family members bind Fibronectin and leucine-rich transmembrane protein family members (FLRT), and that this interaction inhibits axon extension and cell migration by Unc5 expressing cells. FLRT proteins have been previously implicated to regulate neurite outgrowth, cell sorting and adhesion, in a number of studies. It had been previously demonstrated that Unc5B binds to FLRT3, and argued that FLRT3 and Unc5 mediate cell de-adhesion during Xenopus development (Karaulanov et al Plos One 2009). The current manuscript extends these findings to other members of the Unc5 and FLRT families, providing evidence for specificity of interaction between different family members. It also provides evidence that that extracellular domains of FLRT1, FLRT2 and FLRT3 are shed from cells by proteolysis, to liberate the protein fragment to diffuse away from its source to function as a long-range diffusible ligand. Additionally, the manuscript describes in vitro assays that provide evidence that FLRT proteins can trigger growth cone collapse, repel axon extension, and that in vivo FLRT2 and Unc5D inhibit neural cell migration in the embryonic mouse cortex. This is a substantial series of studies.

A) Based on in situ hybridization analysis, the authors conclude that there is no Netrin-1 expressed in the cortex, but neither Netrin-3 nor Netrin-4 have been considered. Both of these Netrins bind Unc5s and can trigger a chemorepellent response (Qin et al 2007 MCN 34, 243-250; Yin et al 2000 MOD 96, 115-119; Wang et al 1999 JN 19, 4938-4947).
Related to the issue of whether or not there is Netrin in the developing cortex, can the authors rule out the possibility that FLRTs bind Netrins. Notably, Yebra et al (2003, Dev Cell 695-707, Fig 1) provides evidence that Netrins bind to Fibronectin, although the interacting domains were not mapped. If FLRTs bind netrin, FLRT binding to Unc5 might be indirect, or cooperative with Netrin binding.
If Netrin protein is absent from developing cortex, this raises important cell survival issues for Unc5B expressing cells. If there is no netrin protein in the cortex, Unc5 expressing cells are predicted to die due to the proapoptotic dependence function of Unc5s. Is it possible that FLRT a dependence ligand for Unc5 that supports cell survival?
Related to cell survival mechanisms, the authors increase the expression of Unc5D by in utero electroporation (Fig 8G,H) and follow the migration of the cells. It is observed that cells expressing increased levels of Unc5D are absent from the upper layers of the cortex and the conclusion drawn that this is due to increased levels of Unc5B inhibiting the migration of these cells. This experiment is confounded by Unc5B functioning as a proapoptotic dependence receptor. In the absence of netrin, increased levels of Unc5B are predicted to kill these cells, which would then reduce the number of cells detected. To conclude that the defect detected is due to an effect on migration, controls must be included to rule out the possibility that the cells are missing from upper cortical layers because they died on route.

B) The findings presented in Figure 8 provide critical evidence for Unc5 - FLRT function in vivo. Several elements of the analysis of the FLRT2 null mice described on page 10 are presented as "data not shown". It would increase the confidence of the reader if at least some of this data was presented, even as a supplemental file if necessary. In particular, the manuscript reports that the number of svet1-positive cells did not change in FLRT2 null mice, but the data is not shown. Presenting a robust cell count of these cell numbers would make this conclusion more compelling. The effects on cell migration detected in vivo in Figure 8 are modest and the images provided to illustrate the differences detected are not particularly compelling. The pairs of sections presented in panels 8A and B, and 8D and E, don't match exactly. For many analyses they might be "close enough", but when justifying the relatively modest change reported here, the differences visible in

the number of cells in the more superficial layers do not engender confidence.
An analysis of the distribution of Tbr+ cells, which are normally confined to the SVZ, revealed a more broad distribution of Tbr+ cells toward the CP in Unc5D null mice, suggesting that the lack of Unc5D allowed these cells to migrate away from the SVZ. The same method of analysis revealed that FLRT2 null mice do not phenocopy the Unc5D mice. This is described with the statement "suggesting that the phenotype in these mice is somehow milder" (pg 11). This implies that it is inexplicable that the phenotypes don't match, however, the phenotype of the FLRT2 null mice simply does not phenocopy the Unc5D mice. Perhaps these data indicate that only a subset of neurons migrating from the SVZ respond to FLRT2 as a repellent, or alternatively that FLRT2 does not influence migration this early in the migratory path. Can the authors comment on the percentage of the cells that are UNC5D+ and are therefore be expected to respond to FLRT2?
The statistical analysis of the data presented in figure 8 is not completely clear. For each study shown, the value of n is described as the number of animals (3 or 4), but then 1 to 5 sections were collected per animal (described in figure legend). It isn`t clear how these multiple samplings from the same animal contributed to the statistical analysis. Does n = 3 animals mean 3 of each genotype, or 3 total? If an experiment had 3 animals of each genotype and 4 sections were taken from each, were those 4 sections used to generate a mean number per section for that one animal, and then these values used to compare between n = 3 animals of each genotype? Alternatively, if an experiment had 3 animals, and 4 sections were taken from each, does that result in n = 12 in the calculation? This would be problematic statistically, as it would likely artificially reduce the variance. If different numbers of slices were taken from each animal this would further bias the outcome toward that one animal. Clarifying the statistical analysis would be appropriate.

The following 2 sets of statements describing FLRT2 null phenotype presented in figure 8 were taken from different parts of the manuscript. While the authors have some evidence supporting a migration deficit in vivo, the data is not as compelling as implied by the strong conclusion listed last.

"In general, ablation of FLRT2 did not alter cortical layering at postnatal stages and did not cause any obvious neuropathological problems (data not shown)..." and "...The same Tbr2 analysis was done in FLRT2-/- brains but the cell distribution was similar to controls." "Considering the high binding affinity of FLRT2 to Unc5D and the strong repulsive effect of FLRT2 on migrating cells, the defect in cortical migration in the respective genetic mutants seems rather modest."

"Together these findings suggest that FLRT2 represents an important in vivo guidance cue that causes Unc5D+ cells to reside in the SVZ for a prolonged period of time before migrating to the CP."

C) Related to figure 5, for clarity, and because it is critical to the conclusion drawn, the authors should include their evidence that hippocampal neurons express Unc5B and Unc5D (currently data not shown). This is important because the cells in the assays shown respond roughly equally to stripes composed of either FLRT2-Fc or FLRT3-Fc, which are argued to selectively bind Unc5D and Unc5B, respectively. This figure provides important evidence that an FLRT interacting with either Unc5 is sufficient to repel axon extension.

D) In the Introduction, the authors correctly indicate that certain deficits in neuronal migration identified in Unc5C null mice were not found in Netrin-1 null mice. They conclude that this indicates the presence of other ligands for Unc5s. The authors should acknowledge that at least 2 other secreted netrins are expressed in brain, netrin-3 and netrin-4, both of which can repel Unc5 expressing cells. An Unc5 ligand that is not Netrin-1, could still be another member of the Netrin family.

Referee #3 (Remarks to the Author):

Based on the finding that Unc5 receptor family members are predicted to have other ligands in addition to Netrin-1, Yamagishi and colleagues aim to study the binding of and in vivo role of FLRT2/3 binding to Unc5D. They hypothesize that migrating neurons that express Unc5D would sense secreted ectodomains of FLRT2, leading a repulsive effect and a delay in the neurons' migration.

The authors use biochemistry to show that neurons in culture express full-length FLRT2 and FLRT3 and secrete cleaved forms of both proteins. They show that these cleavage proteins are glycosylated by treating protein extracts with glycosidases, and also show that the cleavage of FLRT2/3 can be blocked by incubating neurons with metalloproteinase blockers. By designing several deletion constructs, the authors show that the cleavage occurs in the juxtamembrane region of FLRT2/3. Using an overexpression system, the authors show that FLRT2 preferentially binds to Unc5D, whereas FLRT3 binds Unc5B, although FLRT2 and 1 can also bind Unc5B to a lesser extent. The authors then use a growth cone collapse assay using dissociated cortical neurons to show that both FLRT2 and 3 induce growth cone collapse, while neurons with decreased Unc5B (by siRNA) or Unc5D (genetically) lack this collapse response. By plating neurons on a substrate with alternating stripes of FLRT2 or 3 and control (FC), the authors show that neurons preferentially arborize and migrate away from FLRT2/3 expressing stripes. In vivo, during stages of cortical neuronal migration, unc5d is expressed in layers containing migrating cells whereas flrt2 and FLRT2 protein is shown to be expressed in layers through which these cells must migrate. The authors then show that both FLRT2 null mice and Unc5D null mice have greater number of neurons in upper cortical layers (i.e., neurons migrate faster).

Below are some major and minor concerns:

Major issues:

1. The authors present evidence that neurons secrete FLRT1/2/3 ectodomains and that these ectodomains are active in in vitro assays. The authors go as far as identifying that metallproteinases are important for this cleavage event in vitro. However, it is not clear whether this is important in vivo. In fact, the only proof that ectodomains are shed in the cortex (as opposed to in the brain as a whole) is the antibody staining in Figure 7D, which is not convincing. An alternative method should be used to test FLRT2 protein localization. One possible way of showing this is to use the Unc5D-Fc used in Figure 3 to bind to tissue sections and more definitively show FLRT2 protein localization. Moreover, the shedding of ectodomains does not need to be the only explanation for how the migrating neurons sense the other FLRT-expressing neurons at a distance from the FLRT-expressing cell bodies. For example, the processes of those neurons could extend into the layers containing migrating cells and present transmembrane FLRT proteins to the migrating cells. Since the authors have the ability to do in utero electroporation (as shown in Figure 8G), perhaps they could design experiments to block the shedding of the ectodomains in the cortex and then assess neuronal migration.

1. The phenotype of the Unc5D and FLRT2 null mice does not match. There needs to be more explanation of this fact. Although the authors rule out the possibility of Netrin playing a role in this process, perhaps there may be other ligands that play a role in the process. Compound mice could be made to test this hypothesis.

2. Although the in vivo data that the authors examine is cortical migration, none of the in vitro functional assays reported in the manuscript address cortical migration. The growth cone collapse experiments were done in cortical neurons, but growth cone collapse is not the correct experiment to use to draw conclusions about migration. The stripe assay, which includes analysis of cell migration, was done in hippocampal neurons, and is thus not clearly translatable to the cortex. The authors should re-do the stripe assay using cortical neurons.

Minor issues:
2. There are several issues with the western data presented in the paper.
a. The blots are generally very dirty. For example, in Figure2B, the ECD antibody barely recognizes any specific bands at the later ages. It is not explained whether this is a degradation issue, and technical issue, or truly something that happens in vivo. This is in sharp contrast to the ICD antibody in the same figure, which barely recognizes any protein at the later ages (despite there being plenty of protein, if one is to believe the ECD data), and recognizes totally different-sized bands in the null mouse. This type of issue is prevalent throughout Figures 1 and 2.
b. Many of the blots have multiple arrowheads, and the identity of each of these is not clearly explained in the figure legends.

c. In Figure 2, please add markings indicating full length FLRT to the top panels.

3. Figure 4 presents evidence that growth cones of neurons expressing either Unc5B or Unc5D collapse in the presence of FLRT2 or 3. In Panel A, the authors identify a neuron that expresses Unc5B using binding of FLRT3-Fc to this neuron. However, the quantification in D is done with all neurons in the dish (both those that do and do not express Unc5B), which masks the true collapse response. In addition, the siRNA knockdown of Unc5B is shown to be incredibly inefficient (panel F), but this appears to be sufficient to completely block the collapse response. Additionally, two of the conditions presented in panel E show transfection of Unc5B DNA into neurons, which would presumably widen the population of neurons expressing Unc5B and lead to greater percentages of growth cone collapse, but this is not seen. The authors could add a lane to their western data in Panel F to see whether neuronal cultures transfected with Unc5B DNA express greater amounts of the protein, and whether their rescue experiment (unc5B siRNA + Unc5B DNA transfection) has wild-type levels of expression.

4. Figure 6: The only two time-points shown in Panel C are from 2 and 6 hours after plating, what happens during the rest of the time the cells are cultured? Since the time-lapse movie was taken 11 hours after plating, the neurons presumably keep migrating away-showing several more time points between the 6 hours in this figure and the 24 hours in Figure 5 would be useful. In addition, the units used to measure soma migration should be standardized in Figures 5 and 6 (pixels on green stripes vs. cells on green stripes).

5. Figures 7C-E need to be co-stained with a marker in order to truly show that the RNA and protein signal is in different places. Also, Major Issue #1 for concerns about the quality of the antibody staining.

1st Revision - authors' response 19 April 2011

Response to referees' comments

Referee #1 (Remarks to the Author):
...
Comments to figures:

Fig 3G: it is unclear which data to which data are statistically significant
This information has been added to panels G (and also to panel S).

Fig 3: the scatchard plots for unc5B with FLRT2 and unc5D with FLRT3 are missing.
The interaction between FLRT2 and Unc5B is significant (as shown in panels G and S), but it is not strong enough to calculate the Kd value with the binding method used here. We provide a new dose-response curve for FLRT2/ECD-AP towards Unc5B in panel I (together with FLRT2/ECD-AP towards Unc5D). Binding of FLRT2 to Unc5B is saturatable and therefore likely to be specific.
In contrast, FLRT3 binding to Unc5D is very weak and we rather consider it non-specific. We have made the corresponding changes to the text and removed the table from Figure S3.

Fig3 M-S: What is 'representative quantifications'?
We agree that this was a misleading expression. We meant to say that these were the results of a representative experiment. At least one another independent experiment was performed and showed similar results. The sentence in the figure legend was rephrased accordingly.

Fig 4A-C: The growth cones are not visible.
The new panels now show an inset with a higher magnification of the growth cones (new panels Bi and Bii).

Fig 4D: What is used here as a control to unc5B-ECD? What is the result when using unc5D-ECD? What is the molar ratio of unc5B to FLRT3 in these competition experiments?
In this set of experiments, we compared FLRT3-Fc (1 and 3ug/ml) alone with the same amounts of FLRT3-Fc together with Unc5B/ECD (6 ug/ml). The molar ratio was 1:10 (1ug of FLRT2 or FLRT3 versus 6ug of Unc5B). We did not use Unc5D/ECD to block the activity of FLRT3, since Unc5D does not bind FLRT3. Instead, we used Unc5D/ECD to block the activity of FLRT2 (panel

H). In this set of experiments, FLRT2-His preincubated with control Fc was compared to FLRT2-His preincubated with Unc5D/ECD-Fc.

We have now included similar Unc5B/D competition experiments in the stripe assays (see below).

Fig 4E: I don't understand the figure. Why is there an increase in growth cone collapse without expression of unc5B? Then, it appears that the unc5B siRNA has no effect on growth cone collapse rate after unc5B expression. What type of neurons is transfected?
We agree that the description of these results was not optimal. The transfected neurons in panels D and E were rat cortical neurons which based on our ISH with mouse cortex should express endogenous Unc5B (see revised Figure S6). This preparation was also used by Hata et al. (2009) to analyze the collapse activity of RGMa which requires Unc5B. We find that these cells respond to exogenous FLRT3-Fc without the need of overexpression of the receptor. The Unc5B RNAi knock down inhibits growth cone collapse induced by FLRT3-Fc (panel E), and this can be rescued by Unc5B overexpression. We have changed the text in the results to make it clearer for the reader.

Fig. 4F: The quantification of knock-down efficiency missing, which might be actually less than 50%?
Quantification was done and the reduction was >60% (this information was added in the figure legend). The same Unc5B RNAi was used in a previous publication (Hata et al. 2009) and shown to be efficient in reducing the levels of Unc5B thereby affecting the response towards RGMa.

Fig. 4G: Only one independent experiment is shown for growth cone collapse of FLRT2/3-Fc on unc5D mutant axons and needs more experiments. Is the difference in collapse rate between wt and unc5D negative axons in response to FLRT2 significant?
We are thankful to the referee for pointing this out. This experiment was repeated and the new panel G contains the average values of two independent experiments each done in triplicates. The difference in collapse rates between wt and Unc5D-/- neurons in response to FLRT2 is now more obvious: while FLRT2 increases the rate of collapse in wt neurons 2.6-fold, the rate of collapse in Unc5D-/- neurons increases only 2.0-fold. The difference in FLRT2-induced collapse between wt and Unc5D-/- neurons is highly significant ($p<0.01$). In contrast, there is no difference in collapse between wt and Unc5D-/- neurons after Fc and FLRT3-Fc stimulation (revised Figure 4G).

Fig 4H: A 'similar effectiveness' can only be demonstrated in a dose-response curve. What is the amount of unc5D-ECD used here what is the molar ratio to FLRT-2-His?
As suggested, a dose-response curve was done comparing the growth cone collapse responses to similar doses of FLRT2-Fc and FLRT2-His. The results which are depicted in a new panel I, showed no significant differences between the differentially-tagged ligands. For the competition experiment shown in panel H, Unc5D/ECD-Fc was added at a similar molar ratio than FLRT2-His (50 nM and 37.5 nM, respectively). This information has been added to the figure legend.

Fig. 5. Stripe assay experiments in the presence of soluble unc5B and/or unc5D are missing to demonstrate specificity. Also, it appears that for the most part the soma of hippocampal cells are repelled from green Fc stripes in control Fc-Fc assays, leading to an apparent discrepancy between picture F and data in G.
The overlay with green is not helpful as it covers most of the neurites.
We agree and have done competition experiments in the stripe assays. When FLRT2/3-Fc proteins were preincubated with the same amounts of the respective Unc5-ECDs, they lost much of their repulsive activity in the stripe assay, indicating that this function depended on the specific interaction with Unc5 receptors. In this set of experiments, FLRT2/3-Fc were preincubated with control Fc and then compared to FLRT2/3-Fc preincubated with Unc5/ECD-Fc and to Unc5/ECD-Fc alone (revised Figure 5).

The picture of the control Fc-Fc assay was not representative and was replaced. The intensity of the green overlay was reduced so that it does not cover the neurites.

Fig. 6. I wonder why in A the number of cells on Fc and FLRT2 stripes is still the same after 2 h, while the time lapse in D shows that cells move much faster, being repelled within a 2 h window twice already.

The series of images in panel D depicts the behavior of a neuron on the stripes in a time window between 11.5 and 14 hours after plating, while pictures of cells in the original panels A and B had been taken at earlier time points (2 hrs). The times indicated in the panels in D were changed to be more precise. Shortly after plating, cells move much slower, probably because they have to resynthesize some of the cell surface proteins required for migration.

To better illustrate the kinetics of segregation between stripes, we added more time points to the graph in panel C (up to 10 hrs) and replaced the images in panels A and B (4 hr time point).

Fig. 7. FLRT2 and unc5D are expressed in different regions of the brain for the most part, I would not call this complementary as there are huge gaps between their expression domains, or at least it does not suggest some regulatory interactions between FLRT2 and unc5D positive cells.
We agree with the statement of the referee and replaced "complementary" by "non-overlapping" throughout the text. We have, however, added more data suggesting interactions between FLRT2 and Unc5D+ cells (see below).

Fig. 8. Crucially, there is an obvious discrepancy in the location of the flrt2 RNA relative to the corresponding protein (Fig 7C and 7D). The authors provide compelling evidence for shedding of the protein, however, the pattern seen in Fig. 7D is not at all what I would expect from the local RNA pattern shown in 7C.
I would be interested to see the in situ data at E19/20 for flrt2 and unc5d.
We consider the FLRT2 immunostaining specific, since the signal is absent in FLRT2-/- sections (panel E) and the signal in the ganglionic eminence is present in ISH and immunostaining (we have added an asterisk in panels C,D). We have also marked the region between CP and SVZ/VZ with a stippled line to help the reader appreciate the presence of FLRT2 immunoreactivity in the SVZ/VZ region which is devoid of FLRT2 mRNA.

To strengthen the shedding point, we have used an alternative method to test FLRT2/ECD protein localization in the developing cortex. We took E15.5 thick (350 um) coronal sections of the cortex and cut along the IZ to mechanically separate the SVZ+VZ from the CP+MZ. We then analyzed the protein lysates by Western blotting as in Figure 2 using FLRT2/ECD-specific antibodies. Since Pax6 is specific for the VZ, Pax6 antibodies were used to validate the successful separation of the two regions. As shown in revised Figure 7F,G, despite the fact that the SVZ+VZ region does not contain any FLRT2 mRNA, the relative amount of the shed species of FLRT2/ECD was higher in the SVZ+VZ than in the CP+MZ region (ratio shed versus full-length is 0.35 in CP versus 0.8 in SVZ+VZ, n=3 independent experiments, $p<0.05$, t-test). The WB bands indicated by arrow (full-length FLRT2) and arrowheads (shed ECD) are specific as shown in Figure 2. These results indicate that the FLRT2/ECD diffuses away from the CP and is found in the vicinity of the SVZ where Unc5D+ cells are located. An alternative explanation for the presence of FLRT2 in areas devoid of FLRT2 mRNA is the fact, that the FLRT2 positive cells located in the cortical plate at E15.5 migrated as well from the ventricular towards the developing cortical plate. In the process of migration they might have left a trail of cleaved FLRT2 in the apical regions.

We provide in situ hybridization data for flrt2 and unc5D at P0 which corresponds to E19/20 depending on the day of birth (Figure 7). The changes in the expression pattern of these genes and their relevance for the phenotype described here is now better explained (results section) and discussed.

Overall, I am not at all convinced that the data presented suggest a model proposed by the authors including a transient downregulation of the molecules investigated during migration. Actually this model should be supported by further antibody-staining for FLRT2-positive/negative cells during the migration of this particular population to the cortex though I am not sure whether antibodies are available so that this population can be identified.
The model is not about migration of FLRT2-positive cells, but rather about Unc5D-positive/negative cells migrating through the layer of FLRT2-positive cells to upper cortical layers. The paragraph discussing the relevance of the changes of Unc5D expression during cell migration has been revised to make this point clearer. We agree that it would strengthen the model, if we could visualize Unc5D+ cells by antibody-staining, but available antibodies to Unc5D do not work on tissue sections.

Referee #2 (Remarks to the Author):

Based on in situ hybridization analysis, the authors conclude that there is no Netrin-1 expressed in the cortex, but neither Netrin-3 nor Netrin-4 have been considered. Both of these Netrins bind Unc5s and can trigger a chemorepellent response (Qin et al 2007 MCN 34, 243-250; Yin et al 2000 MOD 96, 115-119; Wang et al 1999 JN 19, 4938-4947).
We agree that Netrin-3 and -4 expression patterns should be considered and have included in situ hybridization analysis in the developing cortex (Figure S6, new panels K-N). While we could see specific Netrin-3 staining in places like the thalamus, the signal in the cortex was close to background levels. These results are in line with previous published data (Wang and Tessier-Lavigne, 1999; Pueschel, 1999) and suggest that Netrin-3 is not a physiological ligand for Unc5D+ cells in the SVZ at E15.5.

We find that Netrin-4 is expressed in the VZ of the ganglionic eminences, and in the VZ and SVZ regions of the cortex as previously described (Yin et al., 2000). We have discussed the potential role of Netrin-4 in the discussion section: "This expression pattern would be most consistent with a role of Netrin-4 pushing Unc5D+ cells towards the cortical plate. If this scenario were correct, loss of Unc5D should delay migration, whereas overexpression of Unc5D should accelerate migration. However, the converse is true; loss of Unc5D accelerates migration and overexpression of Unc5D delays migration. We therefore conclude that Netrin-4 does not modulate the migration of Unc5D+ cells towards the cortical plate. It is possible that Netrin-4 in the VZ serves to prevent Unc5D+ cells from undergoing apoptosis (Takemoto et al., 2011)."

Related to the issue of whether or not there is Netrin in the developing cortex, can the authors rule out the possibility that FLRTs bind Netrins. Notably, Yebra et al (2003, Dev Cell 695-707, Fig 1) provides evidence that Netrins bind to Fibronectin, although the interacting domains were not mapped. If FLRTs bind netrin, FLRT binding to Unc5 might be indirect, or cooperative with Netrin binding.
We have tested Netrin-1 binding to cells expressing DCC or FLRT2. We find that fluorescently labeled Netrin-1 binds to DCC as expected, but fails to bind to FLRT2 (Figure S3H). We can exclude cooperative binding between netrin-1 and FLRT2 to Unc5D, since Netrin-1 competes with FLRT2-AP for binding to Unc5D (Figure S3F). This data was included in the results section.

If Netrin protein is absent from developing cortex, this raises important cell survival issues for Unc5B expressing cells. If there is no netrin protein in the cortex, Unc5 expressing cells are predicted to die due to the proapoptotic dependence function of Unc5s. Is it possible that FLRT a dependence ligand for Unc5 that supports cell survival?
Related to cell survival mechanisms, the authors increase the expression of Unc5D by in utero electroporation (Fig 8G,H) and follow the migration of the cells. It is observed that cells expressing increased levels of Unc5D are absent from the upper layers of the cortex and the conclusion drawn that this is due to increased levels of Unc5B inhibiting the migration of these cells. This experiment is confounded by Unc5B functioning as a proapoptotic dependence receptor. In the absence of netrin, increased levels of Unc5B are predicted to kill these cells, which would then reduce the number of cells detected. To conclude that the defect detected is due to an effect on migration, controls must be included to rule out the possibility that the cells are missing from upper cortical layers because they died on route.
These are important questions which we addressed with additional experiments. We stained brain sections after in utero electroporation of GFP or Unc5D/GFP with anti-active caspase3 antibodies. We could not find positive signals in both conditions and GFP+ cells did not have fragmented nuclei (n=4 number of sections; revised Figure S7). To quality control the antibody staining, we treated SH-SY5Y cells with 1 mM paraquate to induce apoptosis. Several caspase3 positive cells were detected under these conditions. Moreover, we have not observed a significant decrease in the numbers of Svet1 (Unc5D+) cells in FLRT2-/- brains (n= 3 mice per group, 3-7 sections per mouse; $p>0.05$ t-test; Figure S7). These results indicate that overexpression of Unc5D or genetic deletion of FLRT2 do not increase the rate of apoptosis in the developing cortex. It is possible that the source of netrin-4 in the VZ/SVZ is sufficient to prevent Unc5D+ cells from undergoing apoptosis. We have mentioned this in the discussion.

B) The findings presented in Figure 8 provide critical evidence for Unc5 - FLRT function in vivo. Several elements of the analysis of the FLRT2 null mice described on page 10 are presented as "data not shown". It would increase the confidence of the reader if at least some of this data was presented, even as a supplemental file if necessary. In particular, the manuscript reports that the number of svet1-positive cells did not change in FLRT2 null mice, but the data is not shown. Presenting a robust cell count of these cell numbers would make this conclusion more compelling.
As mentioned above, we have not observed a significant decrease in the numbers of Svet1 (Unc5D+) cells in FLRT2-/- brains (n=3 mice per group, 3-7 sections per mouse; $p>0.05$ t-test). This information is now provided in Figure S7. We have also included the quantification of the number of Satb2 positive cells in the cortex in control and FLRT2-/- brains (Figure S7).

The effects on cell migration detected in vivo in Figure 8 are modest and the images provided to illustrate the differences detected are not particularly compelling. The pairs of sections presented in panels 8A and B, and 8D and E, don't match exactly. For many analyses they might be "close enough", but when justifying the relatively modest change reported here, the differences visible in the number of cells in the more superficial layers do not engender confidence.
For the quantification of svet1 positive cells in the cortex strict criteria were applied and a careful analysis was performed. Per group, 3 mice were analyzed. To exclude effects on the quantifications through general changes in the cortical architecture on the anterior-posterior axis, the start of the anterior thalamus was used as an anatomical landmark to select sections for analysis. Per group, 6 of these sections were analyzed. To avoid positional effects in the lateral-medial axis of the cortex, areas for quantification were set in close proximity to the border of the ventricular zones of cortex and ganglionic eminence. Pictures were acquired as stacks of 15 images in 2 mm steps to eliminate focal effects. Svet1 positive cells were counted in projections of these stacks. Quantifications of sections grouped by other anatomical landmarks showed a similar shift of svet1 cells towards the cortical plate. However, due to the anatomical changes in the cortex the data could not be pooled. The quality of brightfield stacks of 40 mm sections is in general poor and we apologize for this. Unfortunately our best effort to visualize svet1 or unc5D by immunofluorescence or fluorescent in situ hybridization was not fruitful.

For the analysis of Tbr2 positive cells the same strict criteria were used to select the area of quantification.

An analysis of the distribution of Tbr+ cells, which are normally confined to the SVZ, revealed a more broad distribution of Tbr+ cells toward the CP in Unc5D null mice, suggesting that the lack of Unc5D allowed these cells to migrate away from the SVZ. The same method of analysis revealed that FLRT2 null mice do not phenocopy the Unc5D mice. This is described with the statement "suggesting that the phenotype in these mice is somehow milder" (pg 11). This implies that it is inexplicable that the phenotypes don't match, however, the phenotype of the FLRT2 null mice simply does not phenocopy the Unc5D mice. Perhaps these data indicate that only a subset of neurons migrating from the SVZ respond to FLRT2 as a repellent, or alternatively that FLRT2 does not influence migration this early in the migratory path. Can the authors comment on the percentage of the cells that are UNC5D+ and are therefore be expected to respond to FLRT2?

We estimate that only a small subpopulation of Tbr2+ cells are also Unc5D+. From the svet1 analysis of FLRT2-/- embryos, we know that the migration of a small subpopulation of Unc5D+ cells is influenced by the presence of FLRT2. This FLRT2-sensitive subpopulation of Unc5D+ cells cannot be detected with Tbr2 staining. The fact that Tbr2 staining detects a similar migration phenotype in the Unc5D-/- embryos would suggest that the Tbr2 phenotype in the Unc5D-/- embryos is either independent of FLRT2 or an additive/synergistic phenotype of lack of FLRT2 and of another Unc5D ligand that remains to be identified. We changed the text accordingly.

The statistical analysis of the data presented in figure 8 is not completely clear. For each study shown, the value of n is described as the number of animals (3 or 4), but then 1 to 5 sections were collected per animal (described in figure legend). It isn`t clear how these multiple samplings from the same animal contributed to the statistical analysis. Does n = 3 animals mean 3 of each genotype, or 3 total? If an experiment had 3 animals of each genotype and 4 sections were taken from each, were those 4 sections used to generate a mean number per section for that one animal, and then these values used to compare between n = 3 animals of each genotype? Alternatively, if an experiment had 3 animals, and 4 sections were taken from each, does that result in n = 12 in the

calculation? This would be problematic statistically, as it would likely artificially reduce the variance. If different numbers of slices were taken from each animal this would further bias the outcome toward that one animal. Clarifying the statistical analysis would be appropriate.
We are thankful to this referee for pointing out the lack of clarity in the statistical analysis. In all the experiments of the figure, n refers to the number of animals, not sections. Multiple sections of one animal were used to generate a mean number per section for that one animal, and then these values were used to compare between n animals of each group. We clarified this in the text of the figure legend.

The following 2 sets of statements describing FLRT2 null phenotype presented in figure 8 were taken from different parts of the manuscript. While the authors have some evidence supporting a migration deficit in vivo, the data is not as compelling as implied by the strong conclusion listed last.
"In general, ablation of FLRT2 did not alter cortical layering at postnatal stages and did not cause any obvious neuropathological problems (data not shown)..." and "...The same Tbr2 analysis was done in FLRT2-/- brains but the cell distribution was similar to controls." "Considering the high binding affinity of FLRT2 to Unc5D and the strong repulsive effect of FLRT2 on migrating cells, the defect in cortical migration in the respective genetic mutants seems rather modest."
"Together these findings suggest that FLRT2 represents an important in vivo guidance cue that causes Unc5D+ cells to reside in the SVZ for a prolonged period of time before migrating to the CP."
We agree and have toned down the conclusion:
"Together these findings suggest that FLRT2 represents an in vivo guidance cue that causes Unc5D+ cells to reside in the SVZ for a prolonged period of time before migrating to the CP."

C) Related to figure 5, for clarity, and because it is critical to the conclusion drawn, the authors should include their evidence that hippocampal neurons express Unc5B and Unc5D (currently data not shown). This is important because the cells in the assays shown respond roughly equally to stripes composed of either FLRT2-Fc or FLRT3-Fc, which are argued to selectively bind Unc5D and Unc5B, respectively. This figure provides important evidence that an FLRT interacting with either Unc5 is sufficient to repel axon extension.
We have now included two new panels in figure S6 (E,F) showing the expression of Unc5B and Unc5D in the hippocampus at E15.5 (the same stage used for the stripe assay cultures). While both receptors seem to be expressed in all regions of the hippocampus, highest levels of Unc5D mRNA can be seen in the future CA1 region and highest levels of Unc5B are found in CA3 and the dentate gyrus. From this data we conclude that the population of neurons repelled by FLRT2-Fc is only partially overlapping with that repelled by FLRT3-Fc. In general, FLRT2-Fc is a slightly stronger repellent than FLRT3-Fc which is consistent with the fact that FLRT2 binds to Unc5D and Unc5B, whereas FLRT3-Fc is specific for Unc5B.

D) In the Introduction, the authors correctly indicate that certain deficits in neuronal migration identified in Unc5C null mice were not found in Netrin-1 null mice. They conclude that this indicates the presence of other ligands for Unc5s. The authors should acknowledge that at least 2 other secreted netrins are expressed in brain, netrin-3 and netrin-4, both of which can repel Unc5 expressing cells. An Unc5 ligand that is not Netrin-1, could still be another member of the Netrin family.
We agree and have changed the introduction accordingly.

Referee #3 (Remarks to the Author):
...
Major issues:
1. The authors present evidence that neurons secrete FLRT1/2/3 ectodomains and that these ectodomains are active in in vitro assays. The authors go as far as identifying that metallproteinases are important for this cleavage event in vitro. However, it is not clear whether this is important in vivo. In fact, the only proof that ectodomains are shed in the cortex (as opposed to in the brain as a whole) is the antibody staining in Figure 7D, which is not convincing. An alternative method should be used to test FLRT2 protein localization. One possible way of showing this is to use the Unc5D-Fc used in Figure 3 to bind to tissue sections and more definitively show FLRT2 protein localization.

We agree and have used an alternative method to test FLRT2-ECD protein localization in the developing cortex. We took E15.5 thick (350 um) coronal sections of the cortex and cut along the IZ to mechanically separate the SVZ+VZ from the CP+MZ. We then analyzed the protein lysates by Western blotting as in Figure 2 using FLRT2/ECD-specific antibodies. Since Pax6 is specific for the VZ, Pax6 antibodies were used to validate the successful separation of the two regions. As shown in revised Figure 7F,G, the relative amount of the shed species of FLRT2/ECD was higher in the SVZ+VZ than in the CP+MZ region (ratio shed versus full-length is 0.35 in CP versus 0.8 in SVZ+VZ, n=3 independent experiments, $p<0.05$, t-test), despite the fact that the SVZ+VZ region does not contain any FLRT2 mRNA. The WB bands indicated by arrow (full-length FLRT2) and arrowheads (shed ECD) are FLRT2-specific as previously shown in Figure 2. These results indicate that the FLRT2/ECD protein is found in the vicinity of the SVZ where the Unc5D+ cells are located.

Moreover, the shedding of ectodomains does not need to be the only explanation for how the migrating neurons sense the other FLRT-expressing neurons at a distance from the FLRT-expressing cell bodies. For example, the processes of those neurons could extend into the layers containing migrating cells and present transmembrane FLRT proteins to the migrating cells.
We do not consider this possibility very likely, because we can see a strongly reduced abundance of full-length FLRT2 in the SVZ+VZ region of the cortex where Unc5D+ cells reside (Figure 7F,G). An alternative explanation for the presence of FLRT2 in areas devoid of FLRT2 mRNA may be, that the FLRT2 positive cells located in the CP at E15.5 migrated as well from the VZ towards the developing cortical plate. In the process of migration they might have left a trail of cleaved FLRT2 in the apical regions.

Since the authors have the ability to do in utero electroporation (as shown in Figure 8G), perhaps they could design experiments to block the shedding of the ectodomains in the cortex and then assess neuronal migration.
We agree with the referee that interfering with the shedding of FLRT2 in vivo is an important experiment. There is, however, currently no way to specifically block the shedding of the FLRT2/ECD in the developing cortex. In utero overexpression of non-cleavable FLRT2 may create other cell migration problems and may not yield conclusive results. We are currently generating a (non-cleavable) FLRT2 knock-in allele where the cleavage sequences are deleted. With this allele we will be able to carefully address the contribution of shedding for the in vivo function of FLRT2.

1. The phenotype of the Unc5D and FLRT2 null mice does not match. There needs to be more explanation of this fact. Although the authors rule out the possibility of Netrin playing a role in this process, perhaps there may be other ligands that play a role in the process. Compound mice could be made to test this hypothesis.
We agree that there may be other Unc5D ligands that play a role in this process. We have performed in situ hybridization experiments to rule out the participation of additional ligands of the netrin family (netrin-3 and netrin-4) in this process (see new Figure S6 and discussion). The generation and analysis of FLRT2;Unc5D compound mice turns out to be extremely inefficient due to the partially penetrant embryonic lethal phenotype of the FLRT2 mutants. We have therefore established a conditional FLRT2-lox allele to overcome this problem. The generation of FLRT2-lox;Unc5D compound mice will take 6-9 months.

A sentence regarding the non-matching phenotypes of Unc5D and FLRT2 null mice was added to the text: “The same Tbr2 analysis did not reveal a phenotype in FLRT2-/- brains (data not shown), possibly because the FLRT2-sensitive subpopulation of Unc5D+ cells is too small to be detected with Tbr2 staining. We conclude that the Tbr2 phenotype in the Unc5D-/- embryos is either independent of FLRT2 or an additive/synergistic phenotype of lack of FLRT2 and of another Unc5D ligand that remains to be identified.”
2. Although the in vivo data that the authors examine is cortical migration, none of the in vitro functional assays reported in the manuscript address cortical migration. The growth cone collapse experiments were done in cortical neurons, but growth cone collapse is not the correct experiment to use to draw conclusions about migration. The stripe assay, which includes analysis of cell migration, was done in hippocampal neurons, and is thus not clearly translatable to the cortex. The authors should re-do the stripe assay using cortical neurons.
We agree and have performed additional stripe assays with cortical neurons. These experiments show that FLRT2-Fc is strongly repulsive for cell bodies as well as axons of cortical neurons ($p<0.01$; Figure S5).

Minor issues:

2. There are several issues with the western data presented in the paper.
a. The blots are generally very dirty. For example, in Figure2B, the ECD antibody barely recognizes any specific bands at the later ages. It is not explained whether this is a degradation issue, and technical issue, or truly something that happens in vivo. This is in sharp contrast to the ICD antibody in the same figure, which barely recognizes any protein at the later ages (despite there being plenty of protein, if one is to believe the ECD data), and recognizes totally different-sized bands in the null mouse. This type of issue is prevalent throughout Figures 1 and 2.
We have repeated the blots in Figure 2B with a shorter exposure time so the specific bands of the two shed products and full-length FLRT2 can be easily visualized with the antibodies against the FLRT2-ECD or the FLRT2-ICD used. Furthermore a reduction of FLRT2 expression is visible at postnatal stages. The FLRT2 ICD antibody does not recognize FLRT2 as well as the FLRT2-ECD antibody. This, together with the reduced expression at postnatal stages explains the weak signal for FLRT2-ICD at P0 and P10.

In these new set of experiments the unspecific bands detected in the FLRT2 null tissue disappeared. This was confirmed in a total of 6 experiments using two sets of brain lysates. At E15, little FLRT2 shedding is detected in whole brain extract (Figure 2B), while FLRT2 shedding in cortex is significant (Figure 7F).

b. Many of the blots have multiple arrowheads, and the identity of each of these is not clearly explained in the figure legends.
We have made sure to explain all the arrowheads in the legends.

c. In Figure 2, please add markings indicating full length FLRT to the top panels.
This was done.

3. Figure 4 presents evidence that growth cones of neurons expressing either Unc5B or Unc5D collapse in the presence of FLRT2 or 3. In Panel A, the authors identify a neuron that expresses Unc5B using binding of FLRT3-Fc to this neuron. However, the quantification in D is done with all neurons in the dish (both those that do and do not express Unc5B), which masks the true collapse response.
We agree that the quantification of all neurons (not just those that bind FLRT3-Fc) underestimates the collapse response of the Unc5B+ population. However, Unc5B is well expressed in the cortex at the stage where the tissues were taken for the experiments (see Figure S6). Moreover, it would have been impossible to consider the Unc5B-positive cells using the method in panels A-C in control stimulations (Fc does not bind to neurons) or in the competition experiments with Unc5B-ECD (also Fc tagged).

In addition, the siRNA knockdown of Unc5B is shown to be incredibly inefficient (panel F), but this appears to be sufficient to completely block the collapse response. Additionally, two of the conditions presented in panel E show transfection of Unc5B DNA into neurons, which would presumably widen the population of neurons expressing Unc5B and lead to greater percentages of growth cone collapse, but this is not seen. The authors could add a lane to their western data in Panel F to see whether neuronal cultures transfected with Unc5B DNA express greater amounts of the protein, and whether their rescue experiment (unc5B siRNA + Unc5B DNA transfection) has wild-type levels of expression.
Quantification of the Unc5B knockdown efficiency was performed and turned out to be >60% which apparently was enough to block FLRT3-ECD-induced growth cone collapse (this result was added to the figure legend). The same Unc5B RNAi was used in a previous publication (Hata et al. 2009) and shown to be efficient in reducing the levels of Unc5B thereby affecting the response towards RGMa. Unc5B is widely expressed in the developing cortex and transfection of Unc5B probably does not significantly enlarge the population of Unc5B+ cells. We conclude that the endogenous levels of Unc5B are sufficient to achieve full biological response with the saturating amounts of ligand used in these experiments and that further increases of receptor abundance per cell did not affect the collapse response.

4. Figure 6: The only two time-points shown in Panel C are from 2 and 6 hours after plating, what happens during the rest of the time the cells are cultured? Since the time-lapse movie was taken 11 hours after plating, the neurons presumably keep migrating away-showing several more time points between the 6 hours in this figure and the 24 hours in Figure 5 would be useful. In addition, the units used to measure soma migration should be standardized in Figures 5 and 6 (pixels on green stripes vs. cells on green stripes).
We have quantified several more time points (2, 4, 6, 8, 10 hours) of our time-lapse movies and have added this data to Figure 6C. We have changed the images in panels A and B to better represent the quantifications. These results clearly show that neurons continue to migrate at later stages. The neurons form large aggregates after longer times in culture which prevent us from reliably counting cell numbers past the 10h time point. Life cell imaging is done with phase contrast. Hence, counting cells on stripes is the best method. Stripe assays in which we analyze the behavior of soma and axons are analyzed after Tau1 immunostaining. Counting pixels on stripes is the standard way of quantification. We would therefore prefer to keep the quantifications as they are now.

5. Figures 7C-E need to be co-stained with a marker in order to truly show that the RNA and protein signal is in different places. Also, Major Issue #1 for concerns about the quality of the antibody staining.
We agree with the referee about the quality of the antibody staining, but we believe that the signal is specific since it was completely gone in FLRT2 knock-out tissue. Moreover, our independent micro dissection experiments clearly show that the shed FLRT2 ECDs are enriched in the SVZ+VZ where they can then bind as diffusible ligands to the Unc5D+ cells and induce cell repulsion (Figure 7F,G).

Correspondence 20 May 2011

Thank you for submitting your revised manuscript to the EMBO Journal. The original three referees have now reviewed the revised version and their comments are provided below.

As you can see the referees appreciate the introduced changes. Referees # 1 and 2 are supportive of the revision as is. Referee #3 would appreciate some further in vivo data to support that FLRT2 ectodomain shedding is important. I agree that such data would be nice, but also recognize that this is beyond the scope of this revision. I am therefore pleased to proceed with the acceptance of your paper for publication here. You will receive the formal acceptance letter shortly

Best wishes

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

The authors have addressed the actually quite long list of my initial remarks properly. They also have performed a number of additional experiments to address these concerns.

I think the manuscript is now considerably improved and I fully support publication in the EMBO Journal.

Referee #2

The authors have addressed my comments from the first round of review. The manuscript is improved. I have no further comments or suggestions.

Referee #3

The paper by Yamagishi et al. presents strong in vitro evidence of the effects of FLRT ectodomains on hippocampal and cortical neurons. While in vitro data are convincing, the transfer of the in vitro conclusions to the in vivo situation is more problematic. The experiments that are done in vivo do not rule out other mechanistic possibilities, and while the authors have stated that the experiments proposed by the first round of reviews (e.g., in utero electroporation of a cleavage- resistant form of FLRT2) may provide inconclusive results and were therefore not done, the lack of mechanistic in vivo data hinders the paper's conclusions. They mention that a knock-in mouse expressing a cleavage-resistant FLRT2 is currently being made, and in my opinion, analysis of this mouse will be the gold standard for the authors to make a conclusion about the in vivo role of FLRT2 shedding. However, as stated before, the in vitro data are very compelling. Therefore, whether this paper is suitable for publication in EMBO depends on the Journal's criteria on in vivo evidence.

