A collection of genetic mouse lines and related tools for inducible and reversible intersectional mis-expression

Elham Ahmadzadeh, N. Sumru Bayin, Xinli Qu, Aditi Singh, Linda Madisen, Daniel Stephen, Hongkui Zeng, Alexandra L. Joyner and Alberto Rosello-Diez
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MS TITLE: A collection of genetic mouse lines and related tools for inducible and reversible intersectional misexpression

AUTHORS: Elham Ahmadzadeh, Aditi Singh, N. Sumru Bayin, Xinli Qu, Linda Madisen, Daniel Stephen, Hongkui Zeng, Alexandra L Joyner, and Alberto Rosello-Diez

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. The referees appreciate the amount of work that has gone in to developing these alleles and are convinced by the data presented in the study. All three referees highlight areas where increased clarity in the description of the experiments and results are needed. I think these comments are helpful and addressing them will improve the manuscript. Referee 1 indicates that the Abstract should be more specific and that the Introduction should make clear how the new reagents compare with existing tools. I agree with these comments. Referee 2 makes the important point that the availability of the lines should be indicated. Referee 3 ask for the inclusion of controls from embryos containing DTA alleles, but lacking the tTA allele, to strengthen the evidence that there is no ectopic cell death. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript reports the generation of several new intersectional and reversible mouse lines. The authors report the generation of an inducible cassette that can be turned on and off using rtTA, but only after Cre-mediated removal of tdTom. This allows an intersectional use of lines, as previously reported by the authors, but combines Dox inducibility and, thus, reversibility.

Comments for the author

This reviewer notes and appreciates the large amount of work and investment required to generate new lines and also supports the dissemination of new tools, however, the evidence reported here with these new alleles do not support their use as much more sophisticated, precise means of enacting gene expression changes, nor does it improve in any notable way 'off-target' issues as advertised. The data presented support that the alleles generally work as designed, but do not provide evidence that they have advanced the field with regards to doing sophisticated mouse genetics nor in providing significant new scientific/developmental concepts using the alleles. The data do support a proof of principle in genetic design of these new alleles and some of them may be useful to others in the field.

Comments for author consideration for improving the readability/analyses of the manuscript by readers:

The abstract oversells what is delivered - intersectional use of genetic tools is not new (and previously reported by these authors), nor is Dox, rtTA-driven expression. Combining these is interesting, but the advance in what is offered here over existing tools is overstated. The authors should consider putting more specifics in the abstract regarding the new lines generated for readers who may, indeed, be interested in these new tools. The authors should note the actual tools made here - p21, DTA, Ctgf, etc... and Cres used to highlight this important information to readers.

The introduction, likewise, suggests an increase in specificity and a reduction 'off-target' effects that is not supported. It is not clear to this reviewer what 'off-target' effects of most standardly used Cres are - there are many good Cres available that this reviewer would certainly not characterize as off-target.

Figure 1 legend: the wording of this figure is very hard to follow, even for a mouse geneticist. Particularly confusing is what is meant in the two places where 'with(out)'is used. 'the gene is expressed upon induction with(out) Dox...'

Throughout the text, phrases like "greatly reduced" (page 4, which is 75% reduced it seems), "greatly reduced" (page 6, 60% is arguably not greatly and perhaps should just be reported as a number). For the reporting of new tools, quantitation may be sufficient.

Interpreting figures would be aided greatly by noting the IF stains used in colors in or by the panels that are shown. The induction and collection time should be noted for all figures in the figure or legend in addition to the text.

Page 7, Col2a1-tTA experiment, it was not clear to this reviewer when Dox was added/removed.

Figure 4: The purple color in C does not allow visualization and it is unclear what dashed lines are marking or why this area is the phenotype based on where gene expression is. In D, the control measures zero, but there seems to be TUNEL staining shown. What is quantified in picture in B? And what is the phenotype here - is there one? Should be shown even if not.

In general, more details should be provided in text and legends/figures for readers to follow more easily.

Reviewer 2

Advance summary and potential significance to field

Ahmadzadeh et al in their paper "A Collection of genetic mouse lines and related tools for inducible and reversible intersectional misexpression" expand upon their previously described DRAGON (Doxycline-controlled and Recombinase Activated Gene OverexpressioN) approach for spatial, temporal and reversible control of gene expression. This genetic approach has broad appeal to the developmental biology community and here they have extended its utility by combining with in ovo chick electroporation. The authors have convincingly demonstrated utility of the new DTA model to perform cell ablation studies focusing on neural as well as cartilage development. Additionally, they generated a new line to overexpress Ctgf and perform phenotypic assessment of long bone development using a unilateral approach. These new mouse lines and further characterization of the potential leakiness due to read through make this an appropriate resource article.

Comments for the author

As a resource, it should be made clear where these lines will be deposited since it does not appear that the original lines described in Rosello-Diez et al 2018 have been made publicly available according the Reagents Table. Further, the allele designations of DRAGON constructs should be consistent throughout the manuscript. For example, the Figure 2 legend title contains TigreDragon-DTA but in the figure it is referred to as Igs7LtSl-DTA and in Figure 4 it is referred to as Igs7TRE-LtSl-DTA. Lastly, as a resource, there should be further discussion on its practical usage and potential limitations such as maintenance of a Dragon line. Are the any issues with homozygosity? Have these alleles been crossed on to a common inbred strain background? Have any parent of origin effects been recognized?

In summary, this is potentially valuable resource that can be combined with pre-existing mouse lines to address new questions in developmental biology. The additional validation of the approach in combination with the application to in ovo electroporation overcome some of the novelty concerns related to overlap with Rosello-Diez et al 2018 and Willet et al. 2019.

Minor Comments

(1) A timeline of dox treatment for Figures 2 and 3 would be helpful given main text mentions E13.5 and figure and figure legends are labeled as E13.3

(2) Figure 2 is partially cut-off and staining not clearly labeled in panels A-D

(3) Lines 124-127 and lines 144-147 are highly similar in word content

(4) Figure 4C—Not clear what is being stained

(5) Skeletal preparations mentioned in Fig.5D but no accompanying methods or reagents

(6) Figure 7C difficult to see leaky p21 expressing cells

Reviewer 3

Advance summary and potential significance to field

In the manuscript entitled, "A collection of genetic mouse lines and related tools for inducible and reversible intersectional misexpression", Ahmadzadeh and Singh et al., present the development of a novel genetic system (using both tetracycline responsive elements and loxP sites) to inducibly and reversibly express a given gene of interest. They use this system in two different tissues in mice (cerebellum and limb), as well as in the chicken neural tube. Overall these tools appear to be working well, and thus this work will be of broad interest as a resource for the readership of Development. However, there are some key controls (noted below) that are lacking to truly demonstrate the utility and specificity of these approaches in their different systems. Also, I found

the written descriptions of their early experiments (in particular for Figures 2 and 3) to be quite confusing, especially if taken literally. I would strongly encourage the authors to provide these addition controls, and to consider revising part of the text in their results section to improve their manuscript.

Comments for the author

Major Comments:

1. In Figures 2 and 3, there appears to be a key control missing– mice carrying the Cre allele (either Atoh1Cre in Figure 2, or En1Cre in Figure 3) and the Igs7-DTA allele, but no Atoh1-tTA allele. Presumably, in the absence of tTA, there should be no ectopic cell death observed.
However, the authors should demonstrate that their tool is working as proposed. Similarly, the authors could provide Dox in triple transgenic animals prior to the onset of any Atoh1 or En1 expression to demonstrate no ectopic cell death

2. In Lines 122-127, the authors describe treating with Dox at E13.5 to restrict DTA expression to the eCN, and that this causes ectopic cell death. This is confusing because the data they then show (Fig. 2A-D) are apparently from E13.3 animals (not sure if this is a typo or not), which would be prior to Dox treatment. It also states in the figure legend that these sections are from embryos collected prior to Dox treatment. I think the authors just need to revise their results section to more clearly describe this experiment.

3. In Lines 141-146, the authors use the same confusing language as above, talking about Dox treatment at E13.5 causing ectopic cell death, while showing data from E13.3 prior to any Dox treatment. The text should be revised to more accurately reflect their experiment.

4. In both Figures 2 and 3, I would recommend that the authors quantify the change in cell death between the control and the mutant embryos.

5. The experiment in Figure 4 with the Pitx2Cre is a clever genetic approach; however, the authors should use the same Cre;DTA controls as mentioned above.

6. In Figure 10, the authors should implant beads only (i.e., with no Cre), as well as Cre-coated beads in embryos electroporated with CAGGS-H2B-BFP and TRE-LSL-tdTomato, but not CAGGS rtTA (again, a similar, but important control to validate their tools).

Minor Comments:

1. Part of Figure 2 appears to be cut off (at least in the copy I was provided). I assume this is just a formatting issue that can be easily corrected.

2. The authors should include labels for the IF staining in Fig. 2A-D (presumably green is TUNEL staining, and red is the Tomato reporter).

3. In Figure 2E-F, the authors need to label which is the control and which is the mutant section. Also, Fig. 2G does not distinguish the red vs. black dots (I assume this may be due to the figure being cut off).

4. The lettering as described in the Figure legend for Figure 3 is partially incorrect (for A-D) and should be corrected (e.g., they write “C” when they are actually describing “D”).

First revision

Author response to reviewers' comments

Reviewer 1
Comments for the Author:
1) This reviewer notes and appreciates the large amount of work and investment required to generate new lines and also supports the dissemination of new tools, however, the evidence reported here with these new alleles do not support their use as much more sophisticated, precise means of enacting gene expression changes, nor does it improve in any notable way 'off-target' issues as advertised. The data presented support that the alleles generally work as designed, but do not provide evidence that they have advanced the field with regards to doing sophisticated mouse genetics nor in providing significant new scientific/developmental concepts using the alleles. The data do support a proof of principle in genetic design of these new alleles and some of them may be useful to others in the field.

Comments for author consideration for improving the readability/analyses of the manuscript by readers:

- The abstract oversells what is delivered - intersectional use of genetic tools is not new (and previously reported by these authors), nor is Dox, rtTA-driven expression. Combining these is interesting, but the advance in what is offered here over existing tools is overstated. The authors should consider putting more specifics in the abstract regarding the new lines generated for readers who may, indeed, be interested in these new tools. The authors should note the actual tools made here - p21, DTA, Ctgf, etc... and Cres used to highlight this important information to readers.

The abstract has been extensively revised to be more precise about what is delivered, including specifics of the actual mouse lines that were generated.

- The introduction, likewise, suggests an increase in specificity and a reduction 'off-target' effects that is not supported. It is not clear to this reviewer what 'off-target' effects of most standardly used Cres are - there are many good Cres available that this reviewer would certainly not characterize as off-target.

We think that part of the problem is what the definition ‘off-target’ stands for. What we actually meant is now written in line 70 onwards: “individual promoters/enhancers or gene loci often drive the expression of Cre/(r)tTA in a broader area (or more cell types) than what is required for the experiment, leading to insufficient specificity”. Since the overlapping expression domain of two genetic activators/drivers is by definition smaller or at most equal than any of the individual domains alone, our system directly addresses this issue of insufficient specificity. We now show two examples of the importance of the intersectional approach: the advantage of having the overlap between *En1-Cre* and *Atoh1-tTA* in the cerebellum to remove cells targeted by *Atoh1- tTA* alone in the hindbrain (Fig. 3) and the overlap between *Pitx2-Cre* and *Col2a1-rtTA* removes the expression in the heart, which would be targeted by *Pitx2-Cre* alone (Suppl. Fig. 1).

- Figure 1 legend: the wording of this figure is very hard to follow, even for a mouse geneticist. Particularly confusing is what is meant in the two places where 'with(out)'is used. 'the gene is expressed upon induction with(out) Dox...'

We apologize for the confusion. Since some of our mouse combinations use Tet-On and others Tet-Off approaches, we had attempted to make the legend as general as possible. We now clarify what tools are induced and which ones are shut down by Dox.

- Throughout the text, phrases like "greatly reduced" (page 4, which is 75% reduced it seems), "greatly reduced" (page 6, 60% is arguably not greatly and perhaps should just be reported as a number). For the reporting of new tools, quantitation may be sufficient.

We have removed any qualifiers and stated the quantitative effect instead (page 5 and page 6).

- Interpreting figures would be aided greatly by noting the IF stains used in colors in or by the panels that are shown. The induction and collection time should be noted for all figures in the

figure or legend in addition to the text.

One of the figures was inadvertently cut out during the submission, and hence some labels were missing. We have now made sure that all figures include labels, and that the induction/ collection times are indicated in the legends and/or panels.

- Page 7, Col2a1-tTA experiment, it was not clear to this reviewer when Dox was added/removed.

This has been clarified now. In Fig. 5, no Dox was given. In Fig. 6, Dox was given at E17.5 for shut down experiments.

- Figure 4: The purple color in C does not allow visualization and it is unclear what dashed lines are marking or why this area is the phenotype based on where gene expression is. In D, the control measures zero, but there seems to be TUNEL staining shown. What is quantified in picture in B? And what is the phenotype here - is there one? Should be shown even if not.

We have improved the contrast of this image, to better show the purple color (DAPI). As indicated in the legend, the dashed lines and arrows mark acellular gaps in the cartilage. Panel D shows quantification of TUNEL at P1, whereas panel B shows TUNEL at E15.5. There is a phenotype indeed, which is a remarkable limb asymmetry at P1, due to reduced left-bone growth. We now show and quantify this phenotype in the figure.

- In general, more details should be provided in text and legends/figures for readers to follow more easily.

We have revised the legends to comply with this requirement (especially Fig. 3-5 and 7).

Reviewer 2

- As a resource, it should be made clear where these lines will be deposited since it does not appear that the original lines described in Rosello-Diez et al 2018 have been made publicly available according the Reagents Table.

We fully agree with the reviewer that the lines should be publicly available. As we now indicate in the Discussion, the lines have been accepted by JAX (stocks 034777-034779) and will soon be shipped to them.

- Further, the allele designations of DRAGON constructs should be consistent throughout the manuscript. For example, the Figure 2 legend title contains TigreDragon-DTA but in the figure it is referred to as Igs7LtSl-DTA and in Figure 4 it is referred to as Igs7TRE-LtSl-DTA.

Thank you for noting this. We have unified nomenclature across the manuscript, to use the *Tigre*$^{Dragon\text{-}X}$ version.

- Lastly, as a resource, there should be further discussion on its practical usage and potential limitations such as maintenance of a Dragon line. Are the any issues with homozygosity? Have these alleles been crossed on to a common inbred strain background? Have any parent of origin effects been recognized?

This is important information indeed, now included in the Discussion: “It should be noted that these lines can be maintained in homozygosity with no apparent phenotype, although after some generations some of the homozygous animals show reduced fertility because of inbreeding. Our labs mostly maintain these lines in an outbred SwissWebster background. No parent-of-origin effects have been observed for these alleles”. Re the inbred strains, we maintained for some time a small colony in partial 129Sv background (two generations). This strain was fully functional, but it is no longer available.

In summary, this is potentially valuable resource that can be combined with pre-existing mouse lines to address new questions in developmental biology. The additional validation of the approach in combination with the application to in ovo electroporation overcome some of the novelty concerns related to overlap with Rosello-Diez et al 2018 and Willet et al. 2019.

Minor Comments

(1) A timeline of dox treatment for Figures 2 and 3 would be helpful given main text mentions E13.5 and figure and figure legends are labeled as E13.3

As requested, a time line has been added (Fig 2B), and the details are clarified in the legend. Embryos were collected at E13.5 and the legend is corrected accordingly.

(2) Figure 2 is partially cut-off and staining not clearly labeled in panels A-D

This issue has been fixed.

(3) Lines 124-127 and lines 144-147 are highly similar in word content

This issue has been addressed.

(4) Figure 4C—Not clear what is being stained

It is DAPI, the new version includes a label.

(5) Skeletal preparations mentioned in Fig.5D but no accompanying methods or reagents

Thank you for noticing this. A protocol is provided now.

(6) Figure 7C difficult to see leaky p21 expressing cells

The reviewer is correct. We have added new panels showing p21 signal exclusively.

Reviewer 3
Major Comments:

1. In Figures 2 and 3, there appears to be a key control missing– mice carrying the Cre allele (either Atoh1Cre in Figure 2, or En1Cre in Figure 3) and the Igs7-DTA allele, but no Atoh1-tTA allele. Presumably, in the absence of tTA, there should be no ectopic cell death observed. However, the authors should demonstrate that their tool is working as proposed. Similarly, the authors could provide Dox in triple transgenic animals prior to the onset of any Atoh1 or En1 expression to demonstrate no ectopic cell death

The reviewer makes an important point. We have included images of the requested controls (only tTA, only Cre and only the Tigre allele) in both figures, to demonstrate that ectopic cell death, as expected, does not occur. We have provided Dox after Cre is active and the eCN are born and show that the granule cell precursors are spared.

2. In Lines 122-127, the authors describe treating with Dox at E13.5 to restrict DTA expression to the eCN, and that this causes ectopic cell death. This is confusing because the data they then show (Fig. 2A-D) are apparently from E13.3 animals (not sure if this is a typo or not), which would be prior to Dox treatment. It also states in the figure legend that these sections are from embryos collected prior to Dox treatment. I think the authors just need to revise their results section to more clearly describe this experiment.

We agree the previous version was confusing and have therefore revised the whole section. We used a Tet-Off approach (tTA active without Dox, inactivated by Dox). *Atoh1-tTA* activity is shut down at E13.5 so that the granule cell precursors that are born after this time point are not killed, and therefore in the cerebellum cell death is restricted to the eCN. E13.5 was chosen as the time

point just before adding Dox when cell death will be detected. As now described in more detail, because En1-Cre is active earlier than Atoh1-Cre, the former kills more eCN and at an earlier stage in the region of the rhombic lip whereas with Atoh1-Cre death occurs after the eCN have left the rhombic lip where Atoh1 is initiated because Cre must delete tdTom to allow expression of DTA.

3. In Lines 141-146, the authors use the same confusing language as above, talking about Dox treatment at E13.5 causing ectopic cell death, while showing data from E13.3 prior to any Dox treatment. The text should be revised to more accurately reflect their experiment.

As stated above, this whole section has been rewritten and clarified.

4. In both Figures 2 and 3, I would recommend that the authors quantify the change in cell death between the control and the mutant embryos.

The number of TUNEL+ cells has been quantified and included in the Figures, as suggested.

5. The experiment in Figure 4 with the Pitx2Cre is a clever genetic approach; however, the authors should use the same Cre;DTA controls as mentioned above.

We agree that this is a very important point. Actually, the controls shown in this figure are *Pitx2-Cre; Tigre*$^{Dragon\text{-}DTA}$ animals (now indicated as PC-DTA in the Figure). As per the tightness of the *Dragon* allele in the presence of just *Col2a1-tTA*, this is shown in Figure 7C (middle panel).

6. In Figure 10, the authors should implant beads only (i.e., with no Cre), as well as Cre- coated beads in embryos electroporated with CAGGS-H2B-BFP and TRE-LSL-tdTomato, but not CAGGS rtTA (again, a similar, but important control to validate their tools).

We have done as suggested. The controls behaved as expected (no tdTom was activated).

Minor Comments:

1. Part of Figure 2 appears to be cut off (at least in the copy I was provided). I assume this is just a formatting issue that can be easily corrected.

The reviewer is correct. This has been amended.

2. The authors should include labels for the IF staining in Fig. 2A-D (presumably green is TUNEL staining, and red is the Tomato reporter).

This has been amended.

3. In Figure 2E-F, the authors need to label which is the control and which is the mutant section. Also, Fig. 2G does not distinguish the red vs. black dots (I assume this may be due to the figure being cut off).

Indeed, this was due to the formatting issue, and has been fixed now.

4. The lettering as described in the Figure legend for Figure 3 is partially incorrect (for A-D) and should be corrected (e.g., they write “C” when they are actually describing “D”).

Thank you for noting this mistake, which has now been corrected.

Second decision letter

MS ID#: DEVELOP/2019/186650

MS TITLE: A collection of genetic mouse lines and related tools for inducible and reversible intersectional misexpression

AUTHORS: Elham Ahmadzadeh, N. Sumru Bayin, Xinli Qu, Aditi Singh, Linda Madisen, Daniel Stephen, Hongkui Zeng, Alexandra L Joyner, and Alberto Rosello-Diez
ARTICLE TYPE: Techniques and Resources Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 1

Advance summary and potential significance to field

This manuscript reports the generation of proof-of-principle use of three related mouse lines for doing intersectional genetics.

Comments for the author

This paper has been improved for clarity and the tools generated are much more rigorously defined. Figures are also much improved. A clear report of these new mouse lines is presented.

Reviewer 2

Advance summary and potential significance to field

The authors have significantly improved the manuscript by updating figures and text. All reagents will also be made publicly available providing a useful resource to the community.

Comments for the author

None

Reviewer 3

Advance summary and potential significance to field

The authors have made significant efforts to include the appropriate controls for their studies and have significantly re-written the manuscript to improve clarity for the reader. The authors also appear to have adequately addressed the concerns of the other reviewers. I appreciate their efforts, and believe it has resulted in a significantly improved manuscript.

Comments for the author

I have no additional suggestions for the authors. I support the publication of this study.