

**Title:**

Knock-In of Murine *Calr* del52 Induces Essential Thrombocythemia With Slow-Rising Dominance in Mice and Reveals Key Role of *Calr* Exon9 in Cardiac Development

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## **Abstract**

Frame-shifting mutations (-1/+2) of the calreticulin (*CALR*) gene are responsible for the development of essential thrombocythemia (ET) and primary myelofibrosis (PMF). The mutant *CALR* proteins activate the thrombopoietin receptor (TpoR) inducing cytokine-independent megakaryocyte progenitor proliferation. Here, we generated via CRISPR/Cas9 technology two knock-in mouse models that are heterozygous for a type-I murine *Calr* mutation. These mice exhibit an ET phenotype with elevated circulating platelets compared to wild-type controls, consistent with our previous results showing that murine *CALR* mutants activate TpoR. We also show that the mutant *CALR* proteins can be detected in plasma. The phenotype of *Calr* del52 is transplantable, and the *Calr* mutated hematopoietic cells have a slow-rising advantage over wild-type hematopoiesis. Importantly, a homozygous state of a type-1 *Calr* mutation is lethal at a late embryonic development stage, showing narrowed ventricular myocardium walls, similar to the murine *Calr* knock-out phenotype, pointing to the C-terminus of *CALR* as crucial for heart development.

## **Article**

### ***Introduction***

BCR-ABL negative myeloproliferative neoplasms (MPN) are clonal malignancies of the hematopoietic system, that are represented by three distinct diseases: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) (1). Mutations of the *JAK2* gene are responsible for almost all PV cases and for over half of the cases of ET and PMF (2-5). Frame-shifting mutations in the last exon of the calreticulin (*CALR*) gene are known to be responsible for about a



third of cases of ET and PMF (6, 7). These mutations in the *CALR* gene translate into mutant CALR proteins harboring a mutant C-terminal domain that is rich in positively charged residues. The new domain lacks the endoplasmic reticulum (ER) retaining sequence KDEL, which is present in wild type CALR. The mutant C-terminal domain has been shown to be responsible for the development of the MPN diseases by rendering the mutant CALR protein capable of interacting with and activating the human thrombopoietin receptor (TpoR) also called MPL (8-15) therefore inducing a persistent JAK-STAT pathway signaling in myeloid and especially in MK progenitors. We have shown that mutations in the exon 9 of the murine *Calr* gene that are homologous to the mutations found in patients result in a mutant murine CALR protein harboring an end-tail with a different amino acid composition, but nearly identical biophysical properties as the human CALR mutants (16). Using CRISPR/Cas9, we have previously engineered homologous mutations in the murine *Calr* gene to generate murine CALR mutant proteins that showed the same oncogenic properties as their human counterparts *in vitro* (16).

In the present work, we now have used the CRISPR-Cas9 approach to induce two type 1 *CALR* mutations in the *Calr* gene of mouse zygotes. Previously published mouse models of *CALR* mutant induced MPNs focused on the full (12, 17) or end-tail (18) human del52 mutant, in retroviral (12), transgenic (17) or inducible knock-in (18) settings, respectively. During the publishing of this work, a mouse del19 mutant CALR model was also published (19). We report here our findings on the phenotype, clonal progression and embryonic development in mice expressing endogenous levels of murine del61 or del52 mutant CALR proteins, as well as the phenotype induced by homozygous state.

## ***Materials and Methods***

### ***CRISPR-Cas9 modification of mouse zygotes***

We have used the same small guiding RNAs as described previously (16). For PAM site #1 (5'→3'): ACAAGCAGGATGAGGAGCAG, For PAM site #2 (5'→3'): AGAAGAAGCTGAGGATAAAG. Both sgRNAs were cloned into the px330 vector (gift from Feng Zhang, Addgene #42230). The px330 vectors were micro-injected into the nuclei of fertilized B6D2 mouse zygotes. For the creation of the del52 mutation, we co-injected a single-stranded oligo DNA as template to achieve homology directed repair (HDR) (5'→3'):

GGCTGCAGAGAAGCAGATGAAGGACAAGCAGGATGAGGAGCAGAGAATAAAGA  
GAATGATGATGACAGAGATGAAGATGAGGACGAAGAAGATGA

Confirmation of the exon 9 sequence was performed by cloning the *Calr* locus into an empty plasmid vector and sequencing transformed bacterial colonies. The selected pups were crossed with C57BL/6 – DBA/2 (B6D2.F1) mice and subsequent generations were selected based on the presence of either the del61 or del52 mutation in the *Calr* exon9 by PCR screening.

### ***Bone Marrow Transplantation***

Bone marrow from donor mice was collected by post-sacrifice harvest of both femurs and tibias. Red blood cell lysis was performed by addition of 4 volumes of ammonium chloride 0.8% to 1 volume of bone marrow cell suspension for 10 min at 4°C. Cells were then washed 2 times in PBS and then counted using a Coulter counter. Approximately 1 million cells in a volume of 200 µL of PBS were injected in the retro-orbital sinus of lethally irradiated recipient mice.

### **GeneScan**

Genomic DNA of transplanted mice was extracted from the blood using the Blood DNA Mini Kit from Qiagen (Antwerpen, Belgium). *Calr* exon 9 was then amplified by PCR using a forward primer coupled to 6-carboxyfluorescein (6-FAM) at the 5' end. PCR products were diluted in nuclease-free water at 50X, before adding 10µl of formamide-Rox mix (400µl formamide +4µl Rox Size Standard) to 4 µl of the PCR product and ran the sample on a 3730xl DNA Analyzer (Applied Biosystems, ThermoFischer Waltham, Massachusetts USA.). Data were analyzed with the Gene Mapper software version 4.0. A ratio between the amplified products of *Calr* was used to determine the allele burden.

### **Peptide-based vaccination of mice**

Four 24 AA-long peptides spanning the murine CALR del52 end-tail were synthesized by solid-phase peptide synthesis (SPPS) using conventional fluorenylmethoxycarbonyl (Fmoc) chemistry on a Symphony synthesizer (Protein Technologies Inc) and characterized by mass spectrometry. All peptides used in this study were purified by reverse phase HPLC to > 98% purity and lyophilized. Peptide stock solutions were prepared at approx. 8 mg/ml in water and stored at -20°C. 150µg of a mix of the 4 peptides diluted 1:1 in IFA was injected subcutaneously in the neck of mice recipient of either *Calr*<sup>WT/WT</sup> or *Calr*<sup>del52(50%)/WT</sup> bone marrow, 2 months post-transplantation. A booster shot was injected 1 month after the first shot.

## ***Results***

### ***Endogenous expression of a type 1 murine CALR mutant protein at a heterozygous state reproduces an ET phenotype in the blood and bone marrow.***

Using CRISPR-Cas9 (20-22) system, we created two knock-in mouse models of *CALR* mutant-induced MPNs. We used the same CRISPR/Cas9 set-up as we did before on cell lines *in vitro* (16). Our first knock-in model has a 61-bp deletion (del61) in the exon 9 of *Calr* (23). Our second knock-in model has a 52-bp deletion (del52) in the exon 9 of *Calr* (**Fig 1A**). Both deletions have been engineered to be located at the homologous position as the patient CALR mutations. We first created the del61 knock-in mouse model, as the del61 mutation did not require homology-directed repair post Cas9 cleavage of *Calr*. Interestingly, a homologous del61 human *CALR* mutation had been already described in the *MARIMO* patient derived cell-line (24-26).

Monitoring of red blood cell (RBC), white blood cell (WBC) and platelet (PLT) counts showed that our *Calr*<sup>del61/WT</sup> knock-in model exhibited in average a 15% increase in PLT levels, but with no difference in RBC and WBC levels. The difference in platelet numbers was statistically significant up to 18 weeks of age, but decreased with ageing of the mice as the *Calr*<sup>WT/WT</sup> control mice slowly increased their PLT levels with age (**Fig 1C**).

We observed a higher difference in PLT counts in our *Calr*<sup>del52/WT</sup> model which maintained in average a 35% increase in PLT levels, when compared to control mice. This difference was maintained during the ageing of the mice and was statistically significant at all ages. There was also a significant increase in total white blood cell count at a later age (52 and 60 week old mice) in the *Calr*<sup>del52/WT</sup> mice. This could be due to the fact that the GCSF receptor can be weakly activated by CALR mutants (8, 12). No significant differences in RBC levels were observed (**Fig 1D**).

Histopathological analysis of the bone marrow from *Calr*<sup>del61/WT</sup> mice did not show any difference in megakaryocyte size or numbers in comparison with *Calr*<sup>WT/WT</sup> littermates (**Fig 2A-B**). On the other hand, analysis of bone marrow from *Calr*<sup>del52/WT</sup> demonstrated the presence of staghorn megakaryocytes that are also in average larger and more numerous when compared to those from *Calr*<sup>WT/WT</sup> littermates (**Fig 2C-D**). We did not observe any signs of myelofibrosis in either model, up until an age of 1 year. In the same way, none of the two models exhibited splenomegaly and no myeloproliferation was observed in the spleens (**Supplemental Figure 1**).

***Calr del61 is less active on the murine TpoR than Calr del52, and murine TpoR is only mildly activated by Calr del52.***

We decided to look further into any molecular differences that might explain why the *Calr*<sup>del61</sup>, when compared to *Calr*<sup>del52</sup>, exhibited a weaker phenotype. To this end, by luciferase assay in transiently transfected HEK cells, we show that the murine CALR<sup>del61</sup> mutant exhibits a significantly lower STAT5 activation than the murine CALR<sup>del52</sup> mutant in presence of murine TpoR (**Figure 3A**). Furthermore, because CALR<sup>del61</sup> is only three amino-acids (glutamine-arginine-isoleucine, QRI) shorter than CALR<sup>del52</sup> (**Figure 1B**), we created two extra mutants: CALR<sup>del61+Q</sup> and CALR<sup>del61+QR</sup> that are intermediates between the CALR<sup>del61</sup> and CALR<sup>del52</sup> mutants. We show that addition of the glutamine (+Q) significantly enhances the STAT5 activation, that an extra addition of arginine (+QR) reverses this enhancement, and that the final isoleucine (+QRI) makes the CALR<sup>del52</sup> mutant significantly more potent than the CALR<sup>del61</sup> mutant (**Figure 3A**). A possible explanation is that the extra glutamine dissociates the negatively charged segment before the new tail (CALR<sup>del61>362\_364:DEE</sup>) from the positively charged residues at the outset of the

del61 mutant sequence (CALRdel61>365\_366:KR) inducing a conformational change of the mutant end-tail that enhances its activity (**Supplemental Figure 2**). Addition of the extra arginine to the CALR del61+Q mutant (leading to CALR del61+QR) might reverse the gain by heightening the positively charge patch (CALRdel61+QR>365\_367:RKR), which can be re-dissociated with the neutral bulky beta-branched residue isoleucine (CALRdel52>365\_368:RIKR) in the CALR del52 mutant, which contains the extra QRI sequence.

Furthermore, we show that the murine TpoR is in general activated in a significantly weaker fashion by the CALR de52 mutant than the human TpoR, and that murine CALR del52 is significantly less active than human CALR del52 in general (**Figure 3B**). We have shown previously that TpoR can adopt different active conformations upstream and within the transmembrane (TM) domain (27, 28), and that the His499 residue of the human TpoR, that is substituted to a Leu in murine TpoR, induces flexibility in the human TpoR upstream of the TM domain (29). We therefore tested activation by CALR mutants of the murine TpoR L<sup>tH</sup> mutant, where the Leu 499 is replaced by a His. This mutant displays stronger STAT5 activation in presence of murine CALR del52 (**Figure 3C**). These data might be relevant for understanding the phenotypes in mice of weakly signaling CALR mutants.

***Crossing of the Calr<sup>del52/WT</sup> mouse with the Mpl<sup>Δ60/Δ60</sup> mouse results in loss of the ET phenotype.***

The *Mpl*(*TpoR*)<sup>Δ60/Δ60</sup> mouse model from F. de Sauvage expresses a truncated TpoR protein, lacking the last 60 amino-acids from the cytoplasmic tail, where major signaling tyrosines are located (30). This model where weaker signaling occurs via TpoR does not show signs of thrombocytopenia by itself but shows decreased levels

of megakaryocytic progenitors and megakaryocytes (30). By crossing these mice with our *Calr*<sup>del52/WT</sup> mouse, we aimed to disrupt the CALR del52 induced TpoR activation by impeding the intracellular signaling. We show that *Mpl*<sup>Δ60/Δ60</sup> *Calr*<sup>del52/WT</sup> mice no longer show a significant increase of circulating platelets. Interestingly, mice that were heterozygous for the *Mpl*<sup>Δ60</sup> and the *Calr*<sup>del52</sup> showed significantly elevated levels of circulating platelets, however lower than those observed with *Mpl*<sup>WT/WT</sup> *Calr*<sup>del52/WT</sup> mice (**Figure 3D**). These results demonstrate that an unimpeded TpoR signaling is critical for the development of an ET phenotype induced by frameshifting mutations of *Calr*.

***ET phenotype from the Calr<sup>del52/WT</sup> mouse model is transplantable and Calr<sup>del52/WT</sup> hematopoietic cells exhibit a slow establishing proliferation advantage***

We assessed whether we could reproduce the ET phenotype in lethally irradiated mice by bone marrow transplantation from a *Calr*<sup>del52/WT</sup> donor. We decided to also include three levels of chimeric transplantations of *Calr*<sup>del52/WT</sup> bone marrow: 80%, 50% and 20% of *Calr*<sup>del52/WT</sup> versus *Calr*<sup>WT/WT</sup>. The ET phenotype exhibiting significant increases in platelet levels was reproduced in recipients of 100%, 80% and 50% of *Calr*<sup>del52/WT</sup> bone marrow in comparison with recipients of *Calr*<sup>WT/WT</sup> bone marrow. Interestingly, recipients of 20% *Calr*<sup>del52/WT</sup> bone marrow did not have increased circulating platelets as compared to recipients of *Calr*<sup>WT/WT</sup> bone marrow. No differences in RBC or WBC levels were observed between all populations (**Fig 4A**). In parallel to monitoring circulating blood cells, we decided to assess whether *Calr*<sup>del52/WT</sup> hematopoietic cells would exhibit a proliferation advantage over *Calr*<sup>WT/WT</sup> cells. To demonstrate this, we extracted genomic DNA from whole blood of reconstituted mice at days 40, 100, 150, 250 and 320 post-transplantation and analyzed the *Calr*

del52 allele burden (**Fig 4B**). A slow establishing proliferation advantage was observed over the five time-points for the recipients of 80% *Calr*<sup>del52/WT</sup> and 50% *Calr*<sup>del52/WT</sup>, with a significant increase at 250 and 320 days post transplantation for both populations. No significant establishment of proliferation advantage was observed for the recipients of 20% *Calr*<sup>del52/WT</sup>, although a slight increasing trend was observed. We therefore demonstrated that hematopoietic cells that are *Calr*<sup>del52/WT</sup> have a slow-increasing advantage over *Calr*<sup>WT/WT</sup> cells.

***CALR mutant proteins are secreted from the bone marrow and are detectable in plasma from transplanted *Calr*<sup>del52/WT</sup> mice.***

Knowing that CALR mutants traffic to the cell surface (8, 15, 31-34), we wondered whether they would be detectable in the plasma of our transplanted *Calr*<sup>del52/WT</sup> mice. We extracted the plasma of our *Calr*<sup>del52/WT</sup> transplanted mice and *Calr*<sup>WT/WT</sup> transplanted mice at 2 months post transplantation and tested the soluble proteins by western blot. We show that CALR del52 is strongly detected in the plasma of mice transplanted with 100% *Calr*<sup>del52/WT</sup> and that the levels went down in mice transplanted with both *Calr*<sup>del52/WT</sup> and *Calr*<sup>WT/WT</sup>. No CALR mutant was detected in *Calr*<sup>WT/WT</sup> transplanted mice (**Figure 5**). We were able to semi-quantify the levels of CALR mutant proteins in the plasma of our mice by comparison to the signal detected when probing known concentrations of recombinant human CALR del52 (**Figure 5, Supplemental Figure 3A-B**).

***Peptide-based vaccination against the mutant CALR end-tail elicits a specific humoral response in *Calr*<sup>WT/WT</sup> but not *Calr*<sup>del52/WT</sup> transplanted mice.***



Next, we decided to test whether we could halt or reverse the progression of the ET phenotype using a therapeutic vaccine approach. We synthesized four 24 amino-acid long peptides spanning the C-domain of CALR del52 (**Figure 6A**). These peptides compose the base of the vaccine that was injected subcutaneously in *Calr*<sup>WT/WT</sup> and 50% *Calr*<sup>del52/WT</sup> – 50% *Calr*<sup>WT/WT</sup> chimeric transplanted mice. We opted for a two-shot vaccination protocol, with a booster shot administered one month after the first shot. We monitored the *Calr* del52 allele burden before vaccination, 1 month after the first shot and 1 month after the booster shot. We show that our peptide-based vaccine was unable to reduce the allele burden of our *Calr*<sup>del52/WT</sup> chimeric mice (**Figure 6B**).

We detected presence of circulating anti-CALR del52 antibodies in 3 out of 7 of our *Calr*<sup>WT/WT</sup> transplanted mice by ELISA (**Figure 6C**). As a control, mice that received placebo shots had no detectable antibodies. Importantly, we did not detect the induction of a humoral response in our *Calr*<sup>del52/WT</sup> chimeric mice. This supports the fact that *Calr*<sup>del52/WT</sup> bone marrow cells can induce tolerance for the CALR mutant antigens.

We find these results relevant for future studies related to immunotherapy experiments in the field of CALR induced MPNs and conclude that *Calr* del52 cells can induce tolerance to CALR mutant antigens in chimeric mice and that a simple peptide-based vaccine was inefficient in inducing an immune response.

### ***Homozygous type 1 Calr mutations are lethal during embryogenesis***

In order to assess whether homozygosity of either *Calr*<sup>del61</sup> or *Calr*<sup>del52</sup> could enhance the observed phenotype, we intercrossed *Calr*<sup>del61/WT</sup> and *Calr*<sup>del52/WT</sup> mice to create *Calr*<sup>del52/del52</sup> and *Calr*<sup>del61/del61</sup> mice. Surprisingly, we never obtained any offspring that was homozygous for either mutation. Hypothesizing that the

homozygous state of a type 1 *Calr* mutation would be lethal, we sacrificed 13-day pregnant *Calr*<sup>del61/WT</sup> and *Calr*<sup>del52/WT</sup> female mice that were coupled with a *Calr*<sup>del61/WT</sup> and *Calr*<sup>del52/WT</sup> male mouse respectively. We confirmed by genotyping that amongst the 13-day old embryos, some embryos were homozygous for the *Calr*<sup>del61</sup> or *Calr*<sup>del52</sup> mutation, showing that the homozygous state is lethal during later embryogenesis (**Supplemental figure 4**).

The previously published wild-type *Calr* knock-out mouse model established by M. Michalak's group (35) demonstrated that complete loss of *Calr* is incompatible with life by impairment of heart development and another similar model (36) reported possible failure of closure of the neural tube causing exencephaly in mid-gestation.

None of our *Calr*<sup>del61/del61</sup> or *Calr*<sup>del52/del52</sup> embryos exhibited exencephaly. However, we observed that both *Calr*<sup>del61/del61</sup> and *Calr*<sup>del52/del52</sup> embryos showed thinner ventricular myocardium walls compared to *Calr*<sup>del52</sup> and *del61* heterozygous embryos (**Fig. 7**). These findings agree with observations of the *Calr* knock-out mouse model (35). Therefore, our data show that loss of just the C-terminus Ca<sup>2+</sup>-binding domain of CALR suffices to reproduce the heart development phenotype of the *Calr* knock-out embryos, and that this region is crucial for heart development.

## ***Discussion***

We report two new knock-in mouse models based on frame-shift mutations of mouse *Calr* gene, knock-in *Calr*<sup>del61</sup> and *del52*. Due to the CRISPR/Cas9 editing of the mouse *Calr* gene, the expression of the oncogenic CALR proteins is entirely endogenous in our mouse models. In a heterozygous state, as it is the case for the vast majority of CALR mutated MPN patients (6, 7, 37, 38), we show that endogenous expression of a CALR type 1 mutant protein is sufficient to induce an ET-phenotype.

While human and mouse CALR coding nucleotide sequences are 88% identical, the two proteins are 94.25% identical (39). The cumulative identity and similarity of the amino acid sequences of the novel tails of -1/+2 frameshift mutants are around 70-75%. However, we have previously shown that the biophysical characteristics of human versus murine -1/+2 frameshift mutants are very similar (16).

An interesting observation is that the *Calr* del61 mutation induces a significantly weaker phenotype than the one observed with the *Calr* del52 mutation. Compared to del52, the del61 mutation removes 3 extra amino acids from the wild-type C-terminus of CALR, at the outset of the novel positively charged tail. This subtle change seems relevant to the oncogenicity of the mutant protein, and suggests that not all type 1 CALR mutants behave identically. At this time, no crystal-structure of the mutant CALR protein is available. This might also be relevant as to the development of a CALR ins5 (type 2 mutation) mouse model, as the activity of such a mutant is already known to be weaker than that of type 1 mutants *in vitro* (8, 16). In the CALR del61 mutated MARIMO leukemic cell line derived from a Busulfan-treated patient with ET (25), cells do not need or exhibit JAK-STAT activation (24, 26). This suggests that leukemic transformation was independent of CALR del61 and the leukemia phenotype might have been the consequence of Busulfan treatment.

Interestingly, our *Calr* del52/WT mouse model shows an ET phenotype with an increased number of megakaryocytes, with presence of staghorn features, and of larger size in the bone marrow, but we never observed the development of myelofibrosis. The human CALR del52 transgenic (17), inducible knock-in (18) and mouse CALR del19 (19) mouse models from Shide, Li and Shide respectively also did not progress to myelofibrosis. Both human CALR del52 mouse models reported a 2-fold increase of circulating platelets where we observed a 1.5-fold increase. The mouse

CALR del19 model also showed only a weak increase of platelet levels (19). This difference in platelet levels, as well as the lack of myelofibrosis in all the models could be related to the fact that the murine TpoR responds in a significantly weaker fashion than the human TpoR to type 1 CALR mutants of the same species, as we show here. Indeed, human TpoR exhibits higher flexibility upstream of the TM domain (27-29), and might adopt a more active interface when binding CALR mutants. Additionally, CALR levels of expression from the endogenous locus may also differ in humans versus mice. Of interest, the human del52 CALR mutant induced a myelofibrosis phenotype in retroviral models after 6 months (12), suggesting that expression differences, qualitative or quantitative impact progression to myelofibrosis.

We also show that *Calr*<sup>del52/WT</sup> cells exhibit a proliferative advantage over the *Calr*<sup>WT/WT</sup> cells after transplantation, which was not seen in the model from Li et al. (18), however this proliferative advantage required 140 days to become significantly detectable. Further studies would be necessary to determine whether this advantage occurs at the hematopoietic stem or progenitor levels.

An experimental immunotherapy approach using a peptide-based vaccine did not lead to a decrease of the allele burden of the *Calr*<sup>del52</sup> mutation in our model. We were able to immunize *Calr*<sup>WT/WT</sup> recipient mice but not *Calr*<sup>del52/WT</sup> chimeric mice against the mutant CALR del52 protein, inducing a humoral response with production of mutant CALR specific antibodies, suggesting that *Calr*<sup>del52</sup> hematopoietic cells can enforce tolerance to mutant CALR antigens, possibly because they would generate *Calr*<sup>del52</sup> dendritic cells (40). Those would be sufficient to induce tolerance at the thymus level in the recipient mice, hindering T-helper mediated activation of B-cells against mutant CALR antigens. Premature B-cells recognizing mutant CALR could also undergo negative selection in *Calr*<sup>del52/WT</sup> bone marrow (41). Moreover, neither

*Calr*<sup>WT/WT</sup> or *Calr*<sup>del52/WT</sup> recipients developed significant cytotoxic T cell responses, indicating that the immunogen was weak (Supplemental Tables 1 and 2). Interestingly, a study showed that a series of mutant CALR peptides might not be loaded and correctly presented onto class-I HLA molecules (42), which themselves might be deficient in CALR mutated cells (32). In order to overcome mutant CALR antigen tolerance, a more immunogenic vaccine strategy would be required, such as viral particles.

The fact that the *Calr*<sup>del52</sup> and *del61* mutation are lethal in the homozygous state is highly interesting. Our knock-in models reproduce the heart embryonic defects seen in the wild type *Calr* knock-out model from M. Michalak's group (35, 43), indicating that loss of the distal portion of the C-domain of CALR is sufficient to disrupt the development of the heart during embryogenesis. This can be interpreted by the fact that the high capacity Ca<sup>2+</sup> binding role of the C-domain of CALR is critical for ventricular myocardium embryogenesis. Loss of this function impairs the intracellular calcium homeostasis and signaling (43). Our results further confirm this hypothesis, by showing that loss of the calcium buffering function alone is sufficient to induce embryonic lethality by impairing ventricular myocardium development. It would be of great interest to examine the role of calreticulin in the human pathologies linked to hypertrophic cardiomyopathy.

In conclusion, we show that knocking-in the murine equivalent of *CALR*<sup>del52</sup> in mice leads to an ET phenotype, that is transplantable, while a much weaker phenotype was induced by another mutant also classified as type 1. When studied in bone marrow transplantation setting, we show that after a period of 140 days the knock-in *CALR*<sup>del52</sup> hematopoietic cells exhibit an advantage over the non-mutated

cells. Such slow-rising advantage might be relevant for the natural history of the disease, in contrast to effects just after irradiation and reconstitution. Finally, we show that our knock-in mice exhibited detectable levels of secreted mutant CALR in plasma, and that *Calr* mutated cells can enforce immune tolerance to mutant CALR antigens, in a peptide-based vaccination immunotherapy approach. Our study could provide a novel model for *in vivo* study of myeloproliferation induced by mutants of CALR.

### **Animal Experimentation**

All research and experiments performed on animals in this work have been pre-approved by the local ethical committee (Université catholique de Louvain), following the declaration of Helsinki ethical guidelines. Agreement #2014/28AVR/202.

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### **Conflicts of Interest**

R.K. and S.N.C. are cofounders of MyeloPro Research and Diagnostics GmbH, Vienna, Austria. The remaining authors declare no competing financial interests.

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## Figure Legends

### Figure 1 – *Calr* del61 and *Calr* del52 mutations induce thrombocytosis in a heterozygous state

**A** Comparison of the del61 and del52 mutations engineered by CRISPR/Cas9 in the exon 9 of the *Calr* gene. The first CRISPR single guide RNA recognition site is underlined in purple. The first PAM is in orange uppercase. The second CRISPR single guide RNA recognition site is underlined is light blue. The second PAM is in green uppercase. **B** Comparison of the mRNA translation from the *Calr* del61 and *Calr* del52 mutation. The mutant end-tail, which starts after residue #364 (green bar) is three amino-acid shorter in CALR del61 when compared to CALR del52 that has an extra QRI (underlined). Negatively charged residues are highlighted in red and positively charged residues are highlighted in blue. **C** Circulating platelets, white blood cells (WBC) and red blood cells (RBC) of *Calr*<sup>del61/WT</sup> mice (in red) compared to *Calr*<sup>WT/WT</sup> mice. p-values comparing the two populations are noted over each timepoint when relevant. Analysis performed on a MS-9.5 analyzer (Melet Schloesing Lab, Osny, France). n=12 mice in each group. Statistics were calculated by unpaired non-parametric two-tailed t-test using the JMP Pro 14 software. Vertical bars represent standard deviation. Dotted lines represent normal ranges according to analyzer manufacturer. Data shown are from one experiment representative of at least two

independent experiments. **D** Circulating platelet, red blood cells (RBC) and white blood cells (WBC) of *Calr*<sup>del52/WT</sup> mice (in blue) compared to *Calr*<sup>WT/WT</sup> mice. p-values comparing the two populations are noted over each timepoint when relevant. Analysis performed on a XN-9000 analyzer (Sysmex, Norderstedt, Germany). n=14 mice in each group. Statistics were calculated by unpaired non-parametric two-tailed t-test using the JMP Pro 14 software. Dotted lines represent normal ranges according to manufacturer. Vertical bars represent standard deviation. Data shown are from one experiment representative of at least two independent experiments.

## **Figure 2 – Bone marrow histology of *Calr*<sup>del61/WT</sup> and *Calr*<sup>del52/WT</sup> models**

**A** Hematoxylin-Eosin stained histological slides of *Calr*<sup>WT/WT</sup> (left) and *Calr*<sup>del61/WT</sup> (right) bone marrow from the sternum of 10-months old mice. **B** Left pane: average numbers of counted megakaryocytes per area of bone marrow of *Calr*<sup>WT/WT</sup> and *Calr*<sup>del61/WT</sup> (red) mice. Megakaryocytes were counted at a fixed zoom on a computer and divided by the area (pixels) of bone marrow that was calculated using Adobe Photoshop. Right pane: The average size of 15 megakaryocytes is represented as a dot for each mouse. Size was calculated using a BX43 Olympus Microscope with a 100X MplanAPO/1.40 oil objective, an Olympus DP26 Camera and the Labsens 1.4 software. For both panes, n = 3 mice in each group. **C** Hematoxylin-Eosin stained histological slides of *Calr*<sup>WT/WT</sup> (left) and *Calr*<sup>del52/WT</sup> (right) bone marrow from the sternum of 14-months old mice. The upper left white panels show increased zoom on selected megakaryocytes to show their features. **D** Left pane: average numbers of counted megakaryocytes per area of bone marrow of *Calr*<sup>WT/WT</sup> and *Calr*<sup>del52/WT</sup> (blue) mice. Megakaryocytes were counted at a fixed zoom on a computer and divided by the area (pixels) of bone marrow that was calculated using Adobe Photoshop, two

slides per mice. Right pane: The average size of 15 megakaryocytes is represented as a dot for each mouse. Size was calculated using a BX43 Olympus Microscope with a 100X MplanAPO/1.40 oil objective, an Olympus DP26 Camera and the Labsens 1.4 software. For both panes, n = 5 mice in each group. **B and D**: Statistics were done by unpaired non-parametric two-tailed t-test using the JMP Pro 14 software. Vertical bars represent standard deviation. Data shown are from one experiment representative of at least two independent experiments.

**Figure 3 – The CALR del61 mutant is less active than the CALR del52 mutant, and the murine TpoR is less activated by human and murine CALR mutants than the human TpoR.**

**A** STAT5 activity induced by murine CALR (mCALR) activation of the murine TpoR by dual reporter luciferase assay in transiently transfected HEK cells, in absence or presence of thrombopoietin (20ng/mL) for 48 hours before cell lysis. Experiment performed a minimum of three times, with 3 biological replicates each. Vertical bars represent standard deviation. **B** STAT5 activity induced by murine CALR (mCALR) or human CALR (hCALR) activation of the murine TpoR (mTpoR) or human TpoR (hTpoR) by dual reporter luciferase assay in transiently transfected HEK cells, in absence or presence of thrombopoietin (20ng/mL) for 48 hours before cell lysis. Experiment performed a minimum of three times, with 3 biological replicates each. Raw values were normalized to that of mTpoR + mCALR WT condition across experiments. Vertical bars represent standard error of the mean. **C** STAT5 activity induced by murine CALR (mCALR) activation of the murine TpoR (mTpoR) WT or the Leu499 to His mutant mTpoR (LtH) by dual reporter luciferase assay in transiently transfected  $\gamma$ -2A cells, in absence or presence of thrombopoietin (20ng/mL) for 24



hours before cell lysis. Experiment performed a minimum of three times, with 3 biological replicates each. Raw values were normalized to that of mTpoR + mCALR WT condition across experiments. Vertical bars represent standard deviation. **D** Circulating platelet levels of *Mpl*<sup>Δ60/Δ60</sup> and *Calr*<sup>del52/WT</sup> mice and their crossings, aged 9 to 15 weeks old. Analysis performed on a MS-9.5 analyzer. Dotted lines represent normal range according to manufacturer. Error bars denote standard deviation. Statistics performed by a Mann-Whitney test using the PRISM 5 software. \* : p<0.05, \*\*\* : p<0.005 and \*\*\*\* : p<0.001. Pool of three independent experiments, n ranges from 10 (min.) to 25 (max.). **A , B and C** Statistics were done by non-parametric multiple comparison using Steel's test with control by JMP Pro 14 software. \* : p<0.05, \*\* : p<0.01, \*\*\* : p<0.005.

#### **Figure 4 –*Calr*<sup>del52/WT</sup> transplantation experiments**

**A** Circulating platelets, white blood cells (WBC) and red blood cells (RBC) in lethally irradiated mice transplanted with 100% *Calr*<sup>del52/WT</sup> (blue, plain), 80% *Calr*<sup>del52/WT</sup> (blue, dotted), 50% *Calr*<sup>del52/WT</sup> (grey, dotted), 20% *Calr*<sup>del52/WT</sup> (grey) or *Calr*<sup>WT/WT</sup> (black) bone marrow. Stars show comparison of 100% *Calr*<sup>del52/WT</sup> with *Calr*<sup>WT/WT</sup> mice (see below). n=5 in each sub-group. Counted using a XN-9000 automatic hematological counter (Sysmex corp.). Dotted lines represent normal ranges according to manufacturer. Vertical bars show standard deviation. Data shown are from one experiment representative of at least two independent experiments. **B** Shown is the average of calculated allele burden of *Calr*<sup>del52</sup> for each transplantation chimerism at a certain timepoint post transplantation: 100% *Calr*<sup>del52/WT</sup> (blue), 80 *Calr*<sup>del52/WT</sup> (blue, dotted), 50% *Calr*<sup>del52/WT</sup> (grey, dotted), 20% *Calr*<sup>del52/WT</sup> (grey) and *Calr*<sup>WT/WT</sup> (black). Significance when comparing the allele burden at day 250 or day 320 compared to that

of day 40 for the same chimerism is shown by a star (\*:  $p < 0.05$ ). Statistics were calculated by non-parametric multiple comparison using Steel's method using the JMP Pro 14 software. Data shown are from one experiment representative of at least two independent experiments.  $n = 5$  in each sub-group. Vertical bars show standard error of the mean.

### **Figure 5 – CALR del52 is detectable at high levels in the blood of transplanted mice**

Plasma proteins from three different mice representative of each transplantation chimerism of *Calr*<sup>del52/WT</sup> (100%, 80%, 50% 20%) and of *Calr*<sup>WT/WT</sup> were subjected to western blot. The first lane shows detection of mutant CALR proteins by the anti-mutant rabbit polyclonal antibody SAT-602 (MyeloPro GmbH, Vienna). Below are the calculated semi-quantification of the concentration of mutant CALR in the plasma of each mouse (see supplemental figure 3) expressed in ng/mL. The bottom lane shows detected immunoglobulin light chains for each mouse, as a loading control of plasma proteins. Data shown are from one experiment representative of at least two independent experiments.

### **Figure 6 – Peptide-based vaccination of mice**

**A** Four 24 AA long peptides were synthesized, spanning the end-tail of the mouse CALR del52 mutant. Shown are the peptide sequences (#8, 9, 10 and 11) aligned to the CALR del52 end-tail sequence. Wild-type AA sequence is in blue, del52 AA sequence is in black. **B** Allele burden determined on the peripheral blood of the 50% *Calr*<sup>del52/WT</sup> – 50% *Calr*<sup>WT/WT</sup> transplanted mice. Pre-shot time-point is at 2 months post transplantation (expected allele burden is at 25%) just before vaccine/placebo

shot, 1 month post-shot timepoint is at 1 month after the first vaccine/placebo shot just before the booster shot and 1 month post booster time-point is 1 month after the booster shot, so 2 months after the first shot. Vertical bars denote standard error of the mean. **C** ELISA performed on plasma extracted from mice 2 months after vaccine/placebo administration (1 month post booster timepoint). Plasma was subjected to each of the peptides present in the vaccine and to BSA as a negative control. A purified antibody of known concentration against CALR del52 was used as a standard (not shown). Black bars represent the mean. **B and C** Shown are the results of one experiment, representative of two independent experiments.

**Figure 7 – Heart defects in embryos homozygous for a type-1 *Calr* mutation.**

**A** Left pane: 5µm thick sagittal cut of a 14.5-day old *Calr*<sup>del61/WT</sup> embryo stained with hematoxylin-eosin. The distance between the two red arrowheads is 120µm. Right pane: sagittal cut of a 14.5-day old *Calr*<sup>del61/del61</sup> embryo stained with hematoxylin-eosin. The distance between the two arrowheads is 90µm. Pictures shown are representative of three embryos for each sub-group. **B** Left pane: sagittal cut of a 13.5-day old *Calr*<sup>del52/WT</sup> embryo stained with hematoxylin-eosin. The distance between the two red arrowheads is 70µm. Right pane: 5µm thick sagittal cut of a 13.5-day old *Calr*<sup>del52/del52</sup> embryo stained with hematoxylin-eosin. The distance between the two arrowheads is 21.5µm. Pictures shown are representative of four embryos for each sub-group.

Figure 1

A

*Calr* wild-type exon 9  
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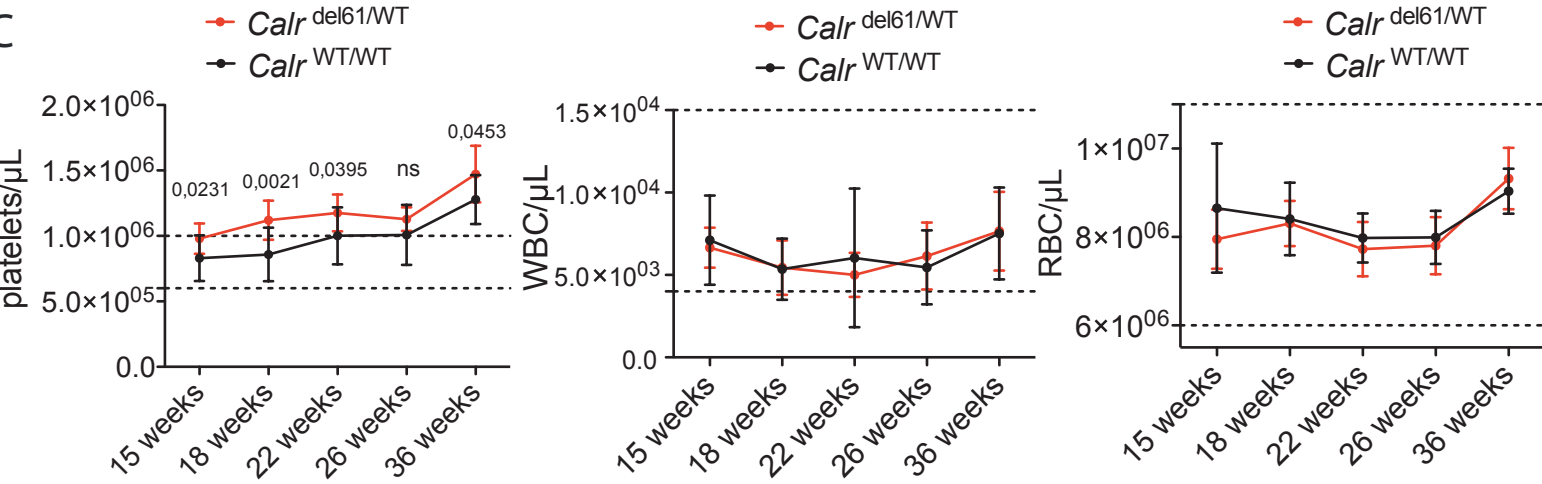
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*Calr* del152 exon9  
5'-aggacaagcaggatgaggagcagAGG-----ataaagAGGatg-3'

B

364  
CALR del161 N' -KQDEE---KRMMMT**EM**KMRTKKMRRRKMRKNPLAKPRMSCRGHTTCLQGWT**EA**\*-C'  
CALR del152 N' -KQDEE**QR**IKRMMMT**EM**KMRTKKMRRRKMRKNPLAKPRMSCRGHTTCLQGWT**EA**\*-C'

C



D

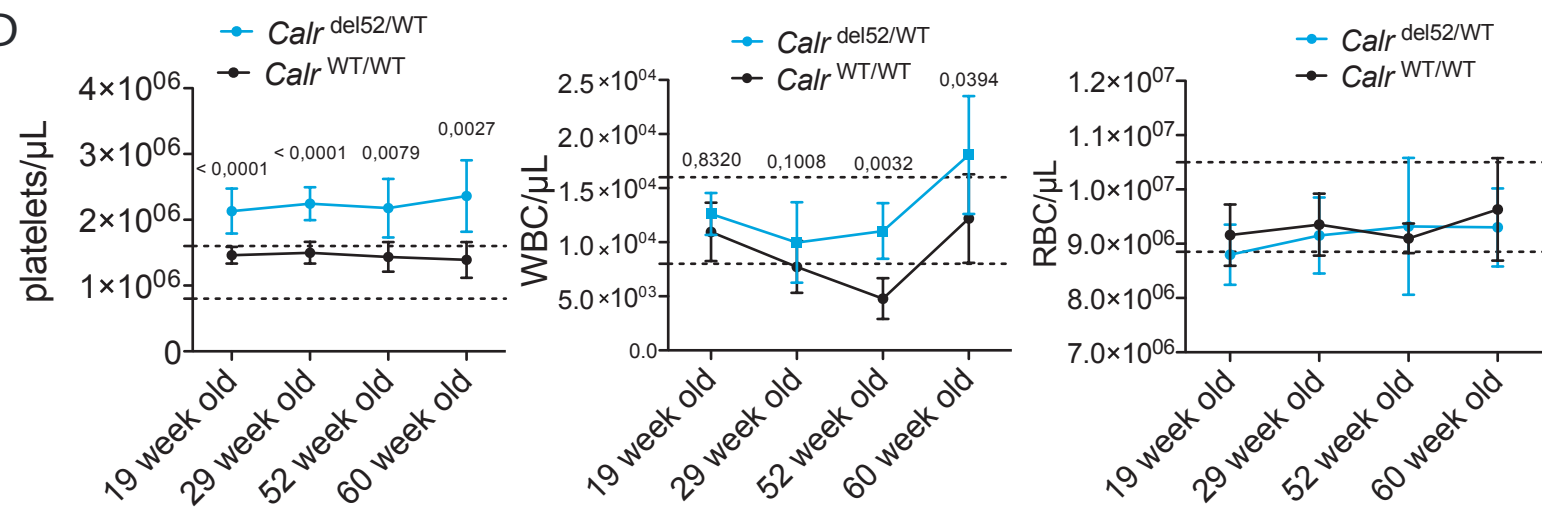
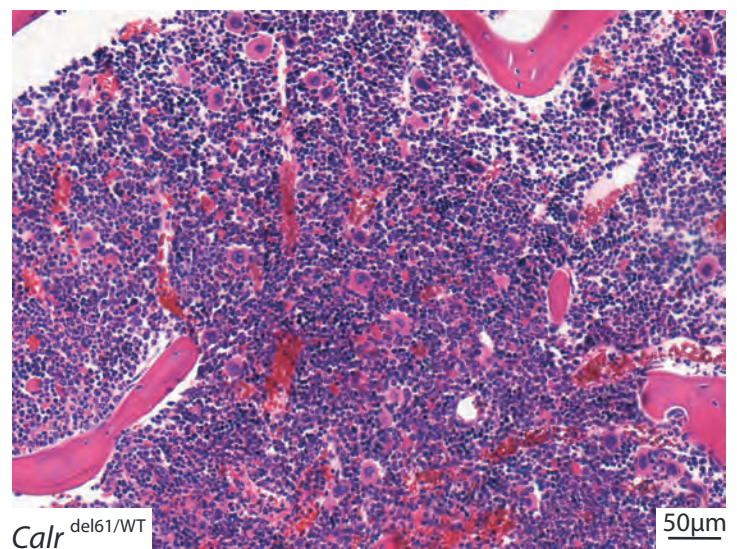
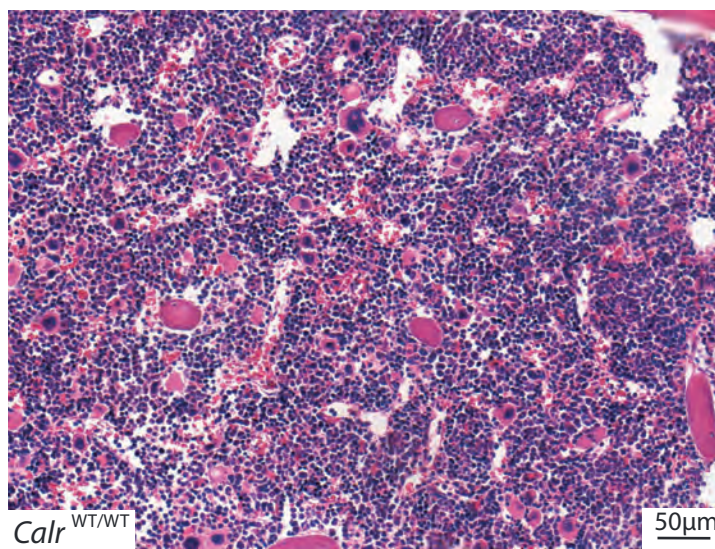


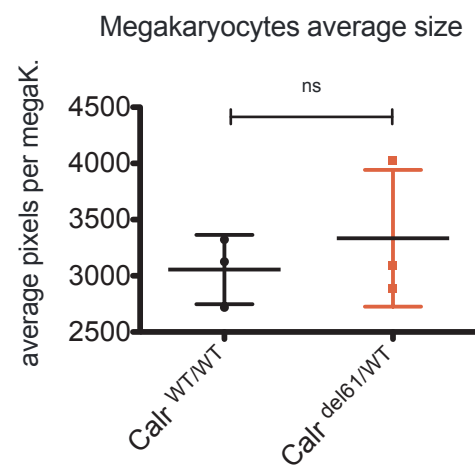
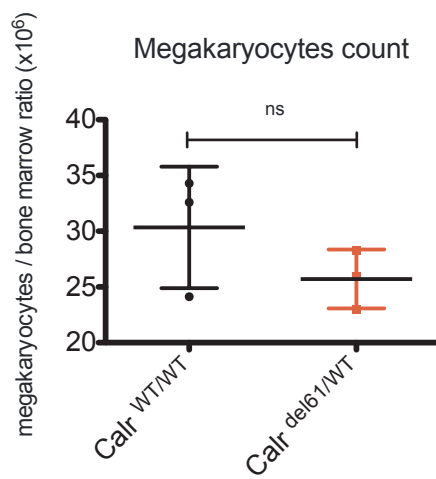


Figure 2

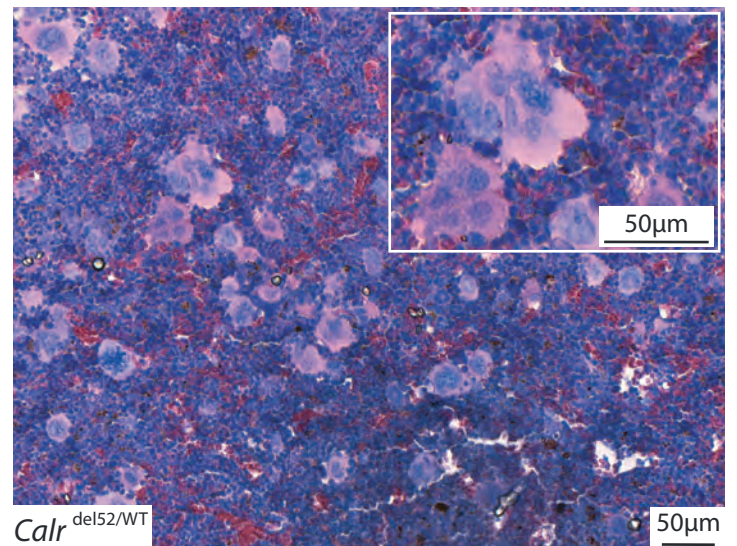
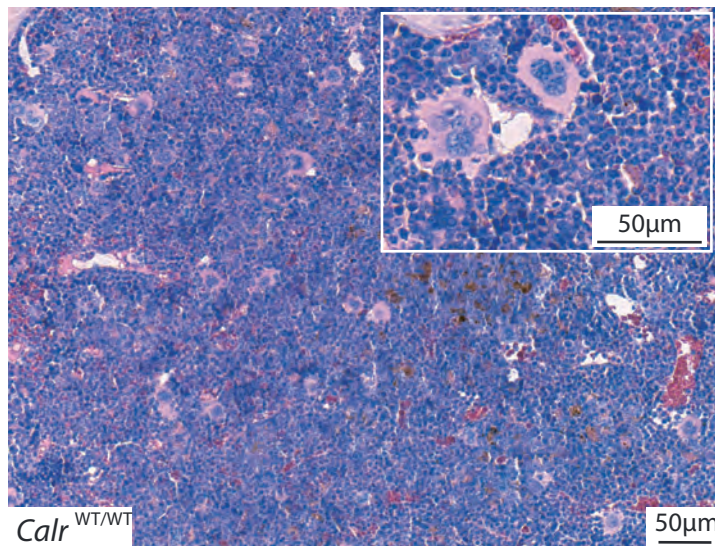
A



B



C



D

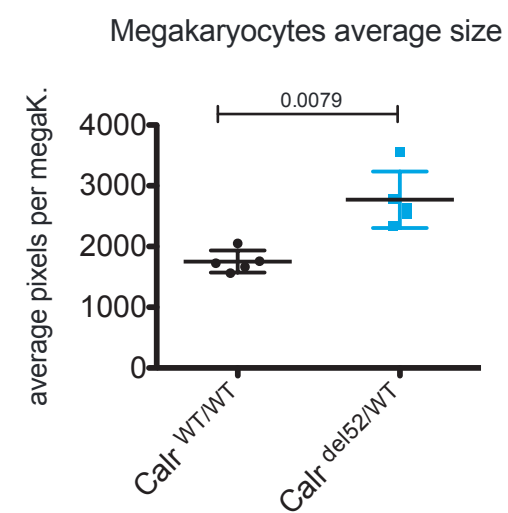
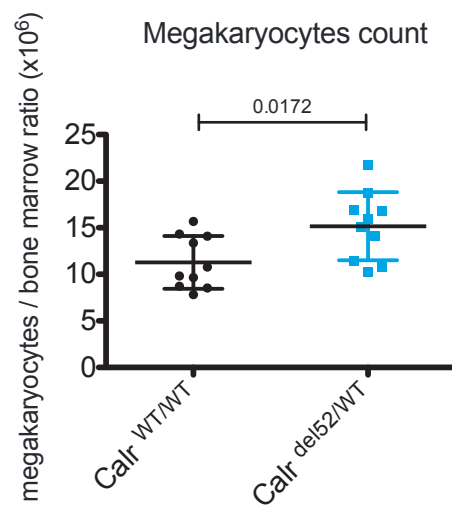


Figure 3

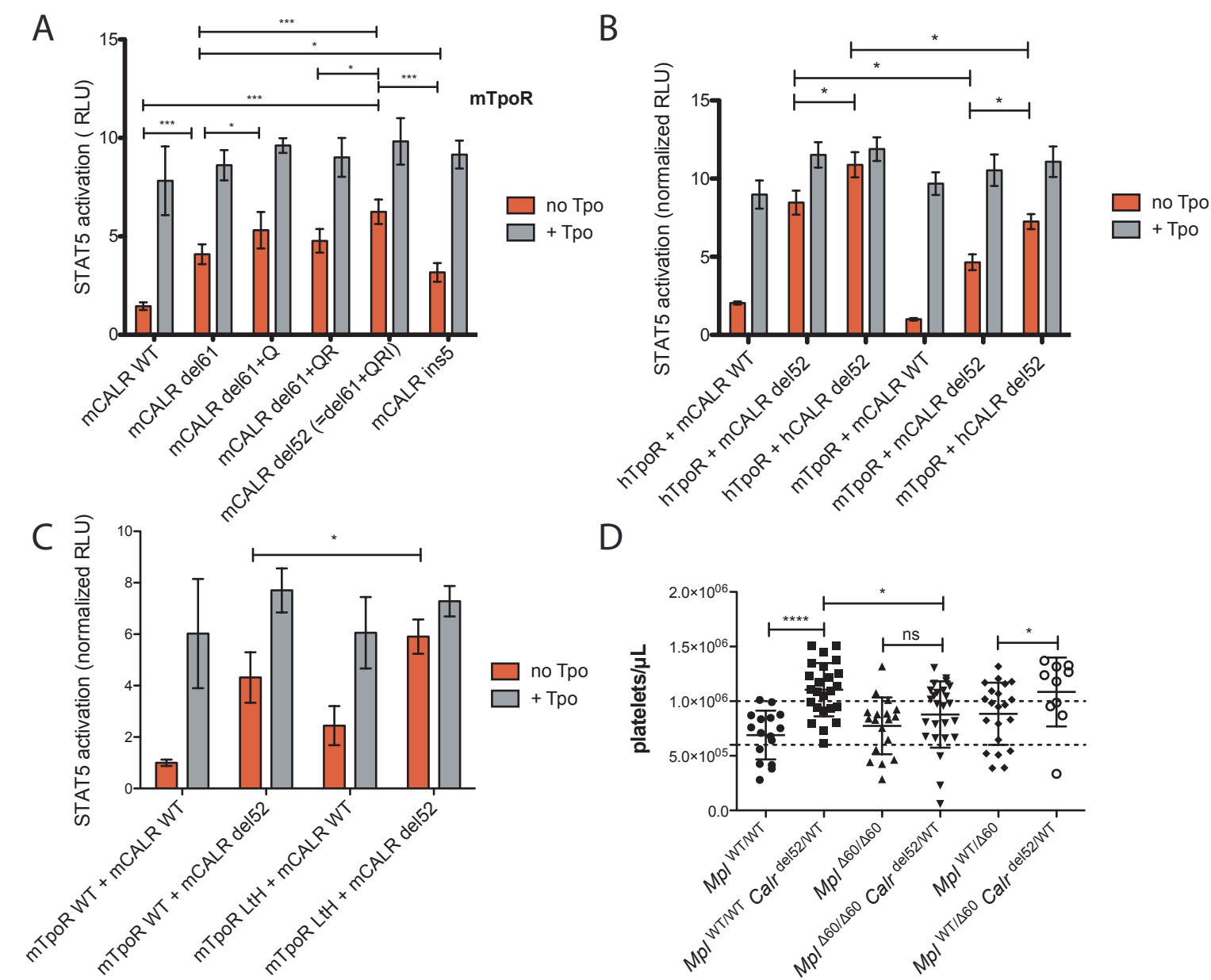
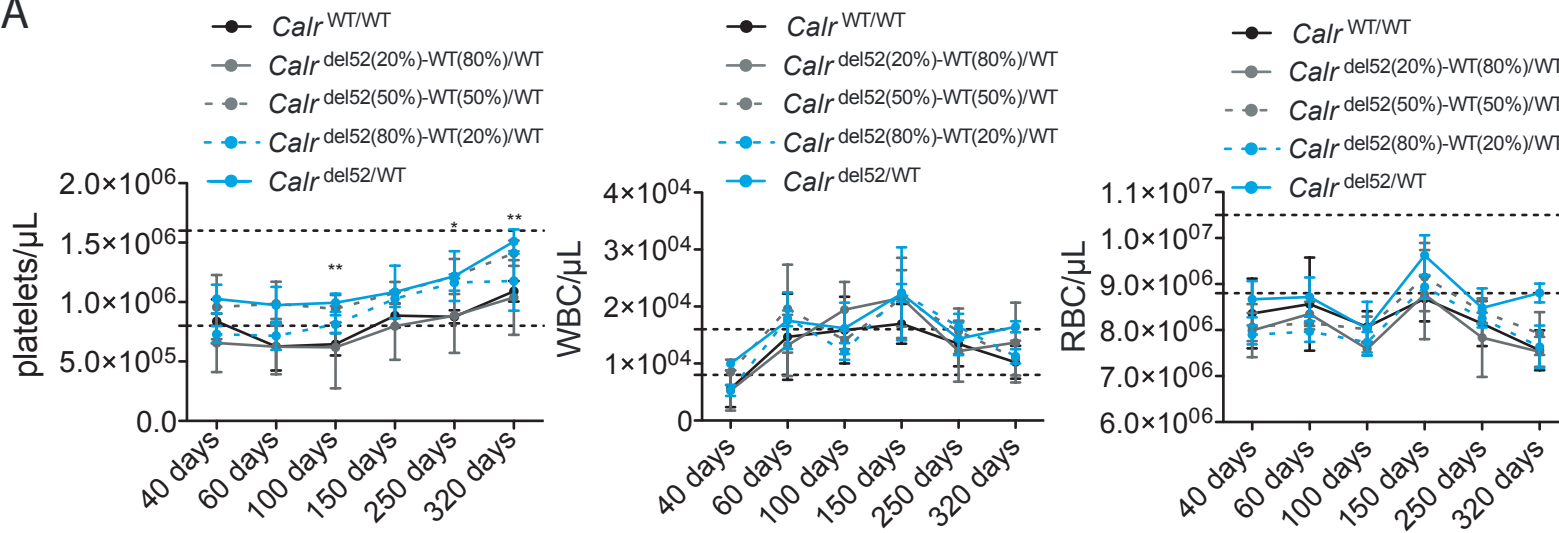


Figure 4

A



B

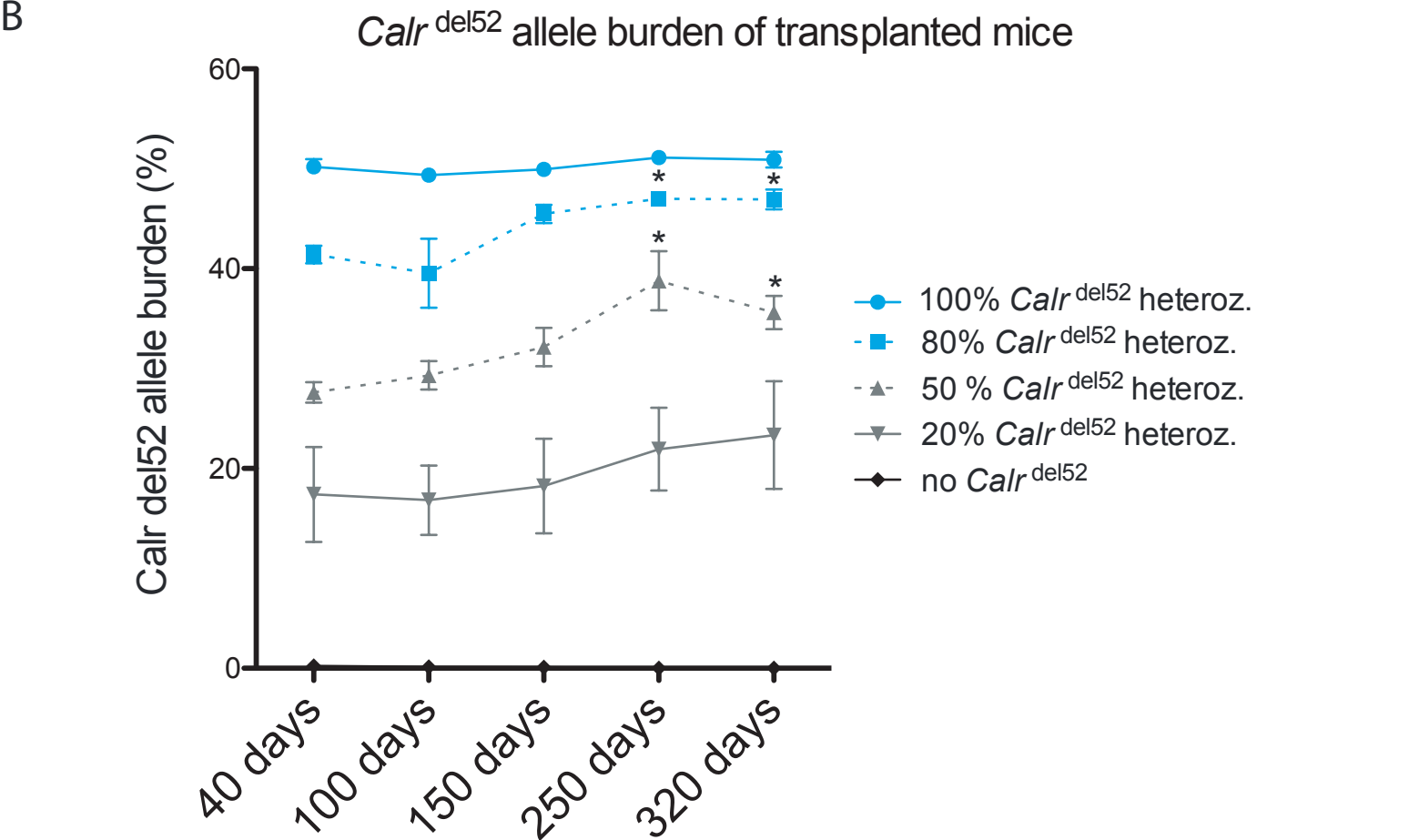




Figure 5

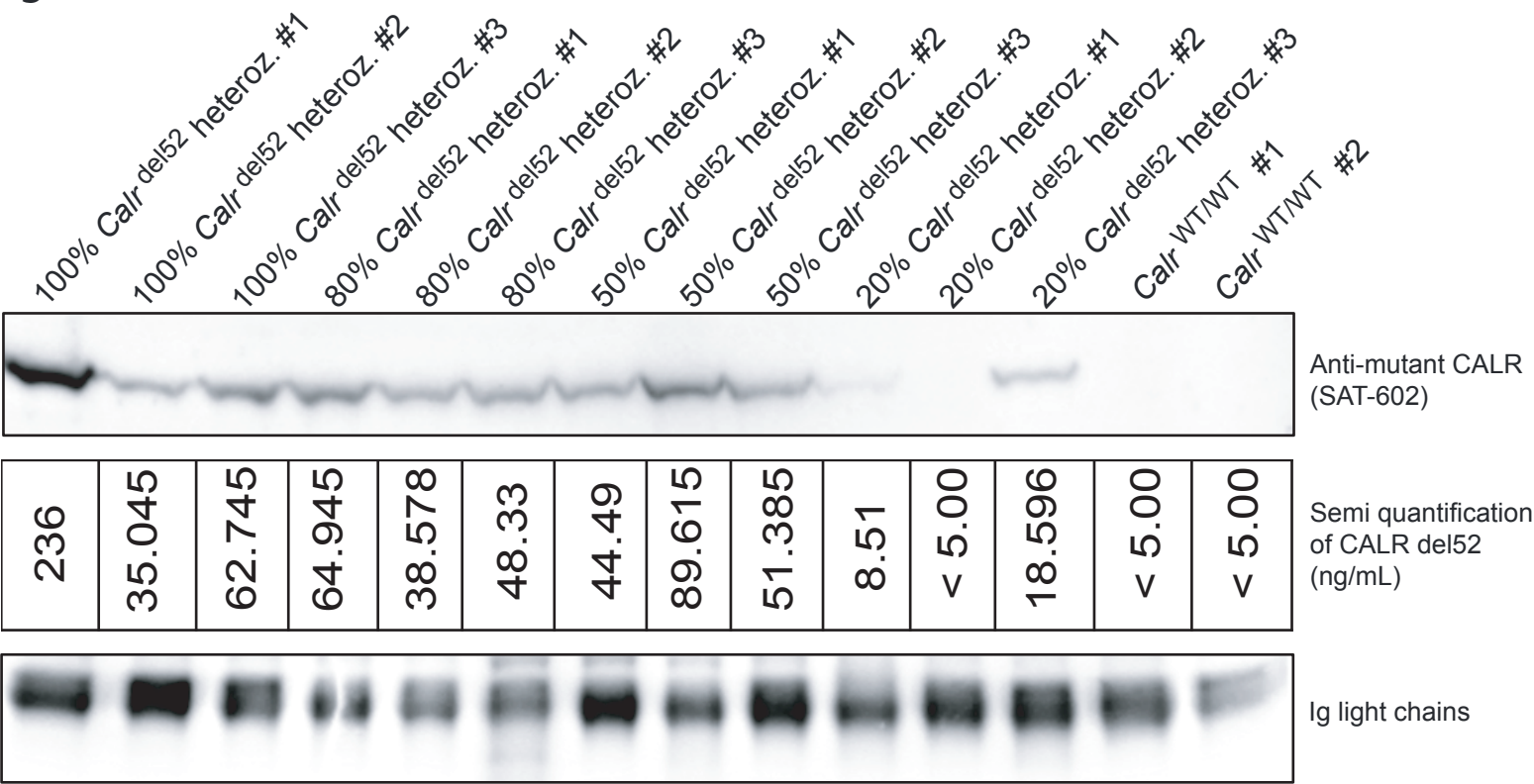
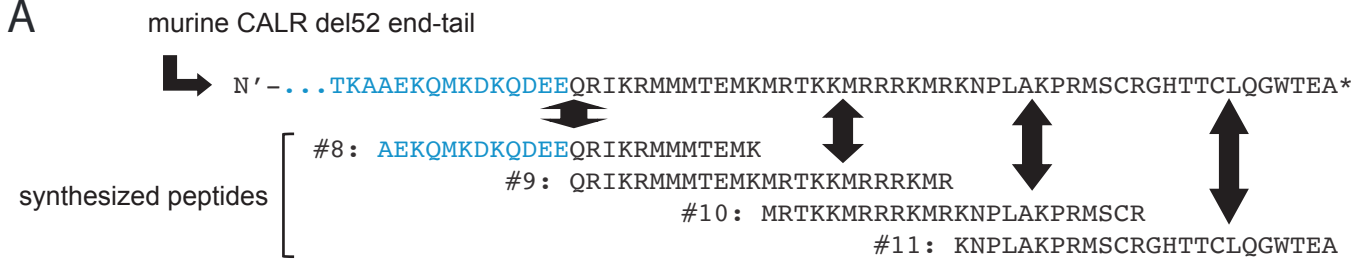


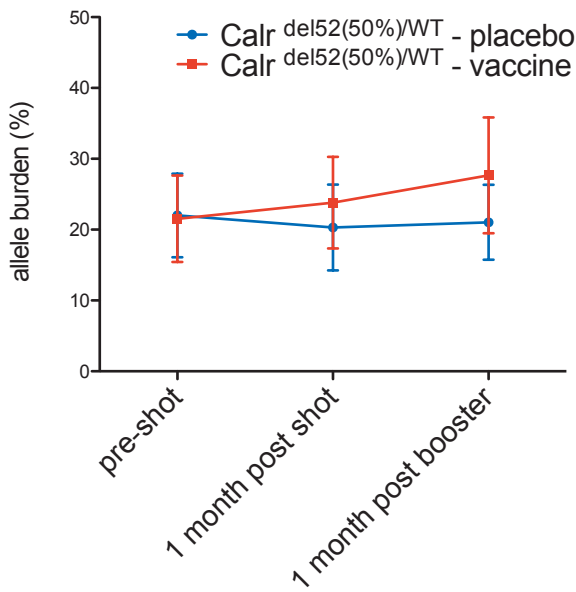


Figure 6

A



B



C

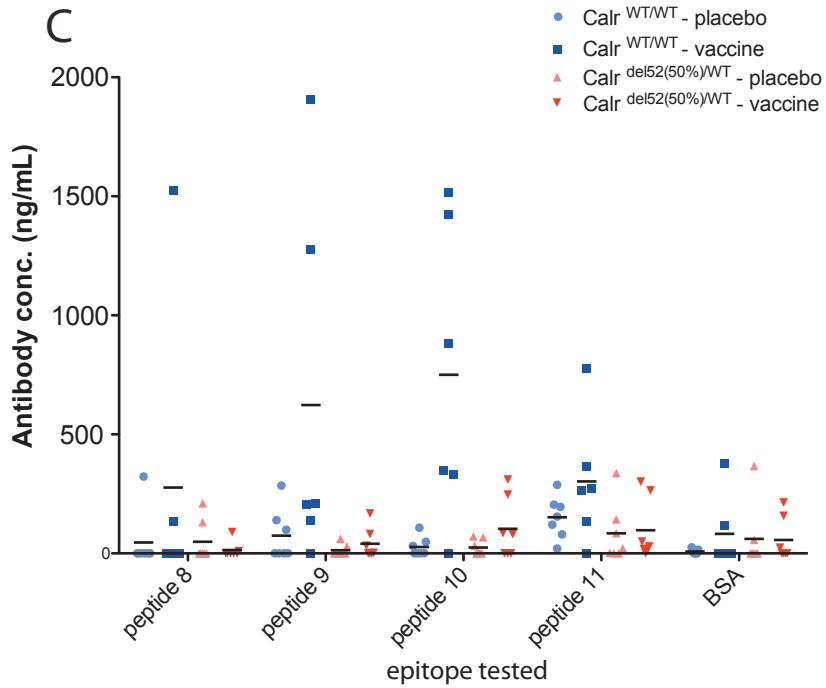
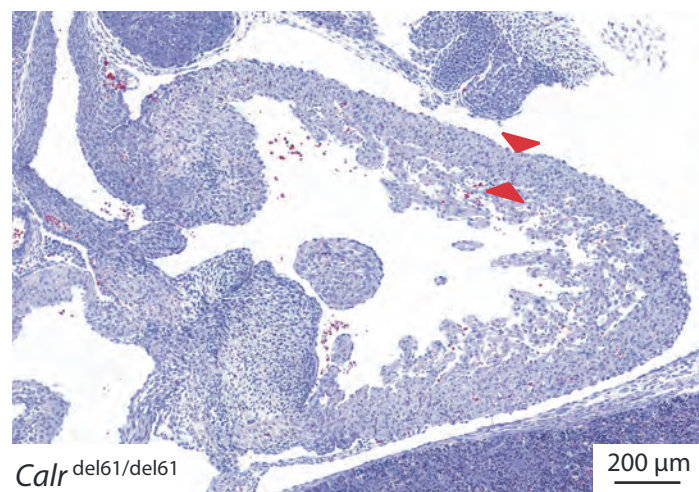
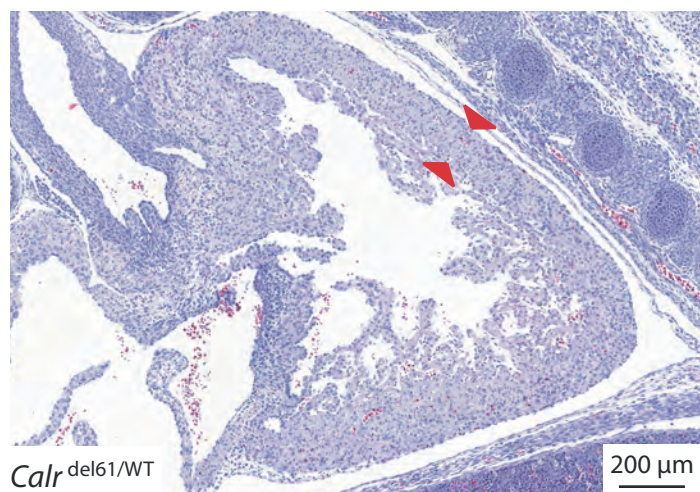


Figure 7

A



B

