The role of nuclear receptor co-activator 4 in erythropoiesis (Reply to Nai *et al.*)

We thank Nai and colleagues for their interest in our recently published paper, Santana-Codina *et al.*,[1] and their comment, also published in Haematologica.[2] Their comment, containing new unpublished experimental data, questions the importance of nuclear receptor co-activator 4 (NCOA4) in acute erythropoietic expansion. Here, we respond to clarify inconsistencies in the interpretation of their new data with respect to our experiments and highlight the multiple lines of evidence that support our overall conclusions regarding the cell autonomous and non-autonomous roles of NCOA4 in supporting murine erythropoiesis. With respect to their concerns over the use of tamoxifen in our inducible model of acute whole body *Ncoa4* genetic ablation: we stand by the conclusions of our experiments as they were tightly controlled with all appropriate genetic backgrounds and treatment controls utilized. While we agree that tamoxifen may have off-target effects on red blood cells (RBC), our experiments as designed and carried out were well-controlled. Specifically, mice in each group had no baseline alterations in systemic iron parameters or erythropoiesis prior to tamoxifen administration making the alterations observed most likely to be a result of acute *Ncoa4* ablation leading to an acute loss of flux of ferritin through the autophagic degradation pathway thereby altering systemic iron homeostasis and basal erythropoiesis. The key difference in the new data presented by Nai and colleagues in their comment[2] is the presence of long-standing *Ncoa4* knockout in their mouse model prior to administration of tamoxifen, which we argue makes their conclusions uninterpretable with respect to our data in an inducible knockout mouse model. Specifically, they use a mouse model with constitutive (from birth) total body knockout of *Ncoa4*, which is a distinct situation compared to acute ablation of *Ncoa4* expression in an adult mice. The critical point here is that with a constitutive total body ablation of *Ncoa4* from birth, erythropoiesis is altered in such a way that RBC at the time of tamoxifen administration already have accumulated long-standing significant changes that alter the baseline response to any agent that has potential deleterious effects on the RBC, such as tamoxifen. For that matter, Nai *et al*. do not present hemoglobin levels in wild- type mice that were subjected to gavage solely with the vehicle (corn oil) that was used to dissolve tamoxifen. Given that repeated gavage is expected to induce a profound stress response, it is unclear if the reduction in hemoglobin levels observed in wild type mice (and which form the basis of their arguments) can be attributed specifically to tamoxifen. While we cannot fully evaluate their claim regarding the lack of alterations in systemic iron parameters in the Sv129/J background they use given their reference to unpublished data, we do note that these *Ncoa4* knockout animals appear to have altered baseline hematologic parameters with microcytosis and a trend towards decreased hemogolobin and hematocrit indicative of at at least a mild alteration in the baseline systemic iron homeostasis and erythropoeisis. As we published in both the tamoxifen-inducible and in our erythroid-targeted *Ncoa4*$^{fl/fl}$; EpoR-Cre model, significant erythroid cell autonomous and non-autonomous compensatory mechanisms are engaged with long-term *Ncoa4* knockout in order to maintain erythropoeisis. Ultimately these compensatory mechanisms are insufficient to restore 'normal' erythropoiesis and therefore erythrocytes are basally altered in long-term *Ncoa4* knockout models. Furthermore, as noted in their original publication of whole body *Ncoa4* knockout, long-term *Ncoa4* ablation leads to a situation of iron overload (including increased serum iron) and predisposes these mice to oxidative stress.[3] Tamoxifen is known to induce oxidative stress.[4] Therefore, we speculate that the effects of tamoxifen in their mouse model are due to an increased sensitivity to oxidative stress in mice with long-standing whole body *Ncoa4* ablation. Our model of acute *Ncoa4* ablation does not have baseline iron overload or a baseline alterations in erythropoiesis and while not formally tested, should not have a baseline increase in sensitivity to oxidative stress, thereby decreasing the possibility of an off-target issue with tamoxifen due to an induction of oxidative stress. In fact, acute ablation of *Ncoa4* should decrease the sensitivity to oxidative stress as has been previously noted by multiple groups.[5–7] We do note that *ex vivo* experiments with erythrocytes from a *Ncoa4*$^{fl/fl}$; EpoR-Cre model were inconclusive with regards to the role of oxidative stress in response to phenylhydrazine; therefore, the formal explanation of tamoxifen effects in mouse models with long-standing *Ncoa4* ablation remains unclear. Regardless, the comparison of tamoxifen administration in a mouse with long-standing *Ncoa4* ablation *versus* a model intended for acute ablation with no baseline alteration in iron or erythroid parameters is fraught with confounders and we argue it is irrelevant.

More broadly, with respect to the role of *Ncoa4* in modulating the response to acute erythropoietic stressors, we refer Nai and colleagues to multiple prior published studies showing that genetic ablation of *Ncoa4* affects erythropoiesis acutely, including our own work in an orthogonal zebrafish model.[8,9] Furthermore, we note our findings in our erythroid specific *Ncoa4* knockout model (*Ncoa4*$^{fl/fl}$; EpoR-Cre) where these animals have an increased sensitivity to acute erythropoietic stress upon phenylhydrazine-induced anemia and have an extra-reliance on compensatory mechanisms to support full recovery of an anemia. These findings all support our conclusions regarding the role of *Ncoa4* in supporting erythropoiesis. Again, we thank the authors for their interest in our publication and on the role of *Ncoa4* in general. We look forward to future collegial discussions and mutual discoveries on the roles of *Ncoa4 in vivo*.

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