Report on a problem studied at the UK Mathematics-in-Medicine Study Group Keele 2012
< http://www.maths-in-medicine.org/uk/2012/stem-cell-pluripotency/ >

The role of E-cadherin in mouse embryonic stem cell pluripotency

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1 Introduction

E-cadherin is a cell-cell adhesion molecule whose function and regulation play critical roles in the suppression of cancer, regulation of tissue growth and epithelial cells [2]. Intracellular proteins regulated by E-cadherin are well documented in the literature and the pathway has more recently been shown to affect the pluripotency of stem cells.

The Study Group participants were asked to focused on understanding how E-cadherin regulates the hierachy of pluripotent signalling pathways in mouse embryonic stem cells. The following questions were posed by the problem presenters

- In which way does E-cadherin interfere with the pluripotency pathways?
- At what stage is E-cadherin most likely to influence Smad-mediated Nanog regulation?
- Can we identify, using a mathematical model of the regulatory pathways, how sensitive our system is to changes in individual parameters?

2 Model Development

We began by first considering a simplified model of the Activin-Nodal and LIF signalling pathways shown in Figure 1 . The Activin-Nodal pathway, as depicted on the left hand side of Figure 2 promotes the production of Nanog via Smad2/3/4, whereas the LIF pathway as shown on the right hand side of the same figure, up-regulates Nanog via Stat3-p.

In order to develop a simplified model of the two pathways (Figure 2), we made the following simplifying assumptions. The signalling pathway which leads to proliferation via FGF2 in the presence of E-cadherin was not included. This is because the focus of our model does not lie on the promotion of proliferation but on the promotion of Nanog. We assumed the

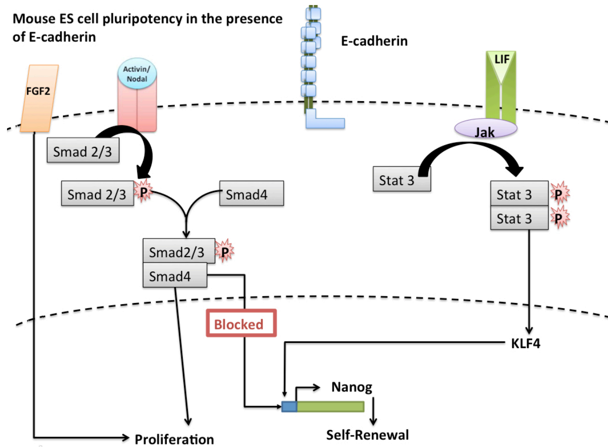


Figure 1: A schematic diagram of the Activin-Nodal and LIF pathways in embroynic mouse stem cells in respect of pluripotency and the effect of E-cadherin.

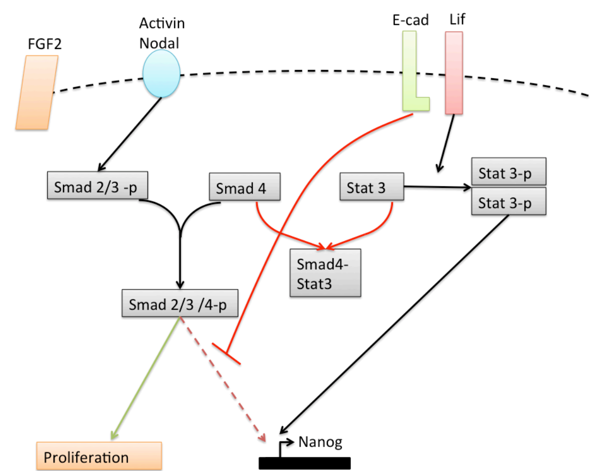


Figure 2: A simplified network diagram of that shown in Figure 1 detailing the key protein-protein interactions which are considered in this report.

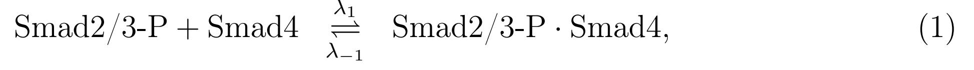
two phosphorylated STAT complex could directly up-regulate Nanog, without the need for a description of KLF4. In order for our model to be able to assist in answering questions (1) and (2) detailed in Section 1 we included a description of the Smad4·Stat3 complex, to account for interactions between the Activin-Nodal and LIF pathways (commonly referred to as cross-tallk) [5], and the inhibition of the Smad2/3/4-promotion of Nanog by E-cadherin.

Although these assumptions are based on experimental findings we know that there is a hierarchy between the two pathways, i.e. with E-cadherin present in the system, the Nanog promotion is realised via the Stat3-pathway. Yet, what is not known is how this hierachy is triggered in reality, i.e. does E-cadherin have a direct or an indirect influence on the pathways, and at which point downstream does the influence occur?

In the work which follows each of the questions presented by the problem presenters were assessed in turn, by first analysing the effects of a possible E-cadherin inhibition, and then investigating the effects of an interlinkage between the two pathways. In Section 2.1 a nonlinear ordinary differential equation (ODE) model of the signalling pathway is developed. A discussion of the model parameterisation is given in Section 2.2. Validation of the mathematical model is discussed in Section 3 and the inhibition of the Activin-nodel pathway by E-cadherin is considered in Section 4.

2.1 Mathematical Model

Figure 2 gives rise to the following reaction equations:



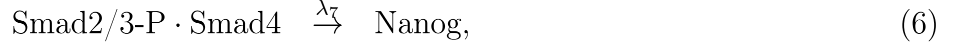
$$\text{Smad2/3-P} + \text{Smad4} \underset{\lambda_{-1}}{\overset{\lambda_1}{\rightleftharpoons}} \text{Smad2/3-P} \cdot \text{Smad4}, \tag{1}$$

$$\text{Stat3} + \text{Smad4} \underset{\lambda_{-2}}{\overset{\lambda_2}{\rightleftharpoons}} \text{Stat3} \cdot \text{Smad4}, \tag{2}$$

$$2\text{Stat3} \underset{\lambda_{-3}}{\overset{\lambda_3}{\rightleftharpoons}} \text{Stat3-p}_2, \tag{3}$$

$$\text{Stat3-p} \overset{\lambda_5}{\rightarrow} \text{Nanog}, \tag{4}$$

$$2\text{Nanog} \overset{\lambda_6}{\rightarrow} \text{Nanog}_2, \tag{5}$$



$$\text{Smad2/3-P} \cdot \text{Smad4} \overset{\lambda_7}{\rightarrow} \text{Nanog}, \tag{6}$$

where each of the reaction rate constants λ_i $(i = 1, -1, 2, -2, 3, -3, 5, 6, 7)$ are assumed positive. We began by assuming that all protein-protein interactions were reversible; a reasonable stating point given many such reactions are reversible in a number of systems. We note that equation (5) represents the self-promotion of Nanog via dimerisation [4].

The Law of Mass Action was applied to equations (1)-(6) to give

$$\frac{dS_4}{dt} = \phi_2(1 - \rho\chi) - \lambda_1 S_4 + \lambda_{-1} S_{234} - \lambda_2 S_4 + \lambda_{-2} S_{34} - d_1 S_4, \tag{7}$$

$$\frac{dS_{234}}{dt} = \lambda_1 S_4 - \lambda_{-1} S_{234} - \frac{\lambda_7 S_{234}}{1 + \mu S_{3p_2}}(1 - \chi) - d_4 S_{234}, \tag{8}$$

$$\frac{dS_3 p_2}{dt} = \chi\phi_4 - \lambda_{-3} S_{3p_2}, \tag{9}$$

$$\frac{dS_{34}}{dt} = \lambda_2 S_4 - \lambda_{-2} S_{34}, \tag{10}$$

$$\frac{dN}{dt} = \frac{\lambda_7 S_{234}}{1 + \mu S_{3p_2}}(1 - \chi) + \lambda_5 \chi S_{3p_2} + \lambda_6 \frac{N^2}{N^2 + K^2} - d_3 N, \tag{11}$$

$$\frac{dP}{dt} = \frac{\lambda_{-2} S_{34}}{1 + \lambda_2 S_4} - d_4 S_4 P \tag{12}$$

where S_4 = [Smad4], S_{234} = [Smad2/3-P $\cdot$ Smad4], S_{3P_2} = [Stat3-p$_2$], S_{34} = [Smad4$\cdot$Stat3], N = [Nanog], ρ is the enhanced Smad4 production when E-cad is OFF, μ is the Threshold-value for S_{3P_2}, K is the maximal level of Nanog effectiveness and χ acts as an ON-OFF switch in order to simulate the system in the presence and absence of E-cadherin, effectively such that

$$\chi = \begin{cases} 0 & \text{if E-cad OFF} \\ 1 & \text{if E-cad ON} \end{cases}$$

We see that we have neither an equation for Smad2/3-P nor for Stat3. This is because experimental data indicates these two molecules are in excess in comparison to others in

the signalling pathway and thus their variation is negligible over the timescale the pathways are activated [1, 3].

Experimental evidence shows that the absence of E-cadherin leads to a fourfold increase in the production of Smad4; this effect is account for by ρ. We also include a sort of threshold value for S_{3p_2}, called μ, which will only be of interest in the later sections of this work, but is mentioned here for the sake of completeness. We implement a capacity function K for the concentration of Nanog which changes its speed of self promotion depending on the present Nanog-concentration.

The system of governing ODEs is closed with the initial conditions

$$S_4 = S_{40}, \qquad S_{234} = 0, \qquad S_{3P_2} = S0_{3P_2}, \qquad S_{34} = 0, \qquad N = 0 \quad \text{and} \quad P = 0, \qquad (13)$$

at $t = 0$.

2.2 Parameterisation

Given time, the difficulty in obtaining data to fully parameterise the governing system of ODEs and in order to be able to understand the qualitative nature of our formulated model, the following assumptions were made in informing the model parameter values. All concentrations were assumed equal to unity. All rate constants were assumed equal to unity with the exception of $\lambda_2 = 0.1$, $\lambda_{-2} = 2$, $\lambda_7 = 5$, $\phi_2 = 2$, $\phi_4 = 3$ and $\rho = 0.8$. These values were informed by discussions with members of the Manchester Stem Cell Group and in obtaining model solutions that exhibited the same general behaviour as that observed experimentally.

In respect of understanding how the presence of E-cadherin affected the Activin-Nodal pathway and Nanog expression, relative levels of the proteins modelled in our pathway were considered. These values are detailed in Table 2.2.

	Smad4	Smad2/3/4	Stat3-p_2	Smad4 $\cdot$ Stat3	Nanog
E-cad off	4	1	0	4	0.5
E-cad on	1	1	1	1	1

Table 1: Relative protein concentrations for each protein in the signalling network in the presence and absence of E-cadherin.

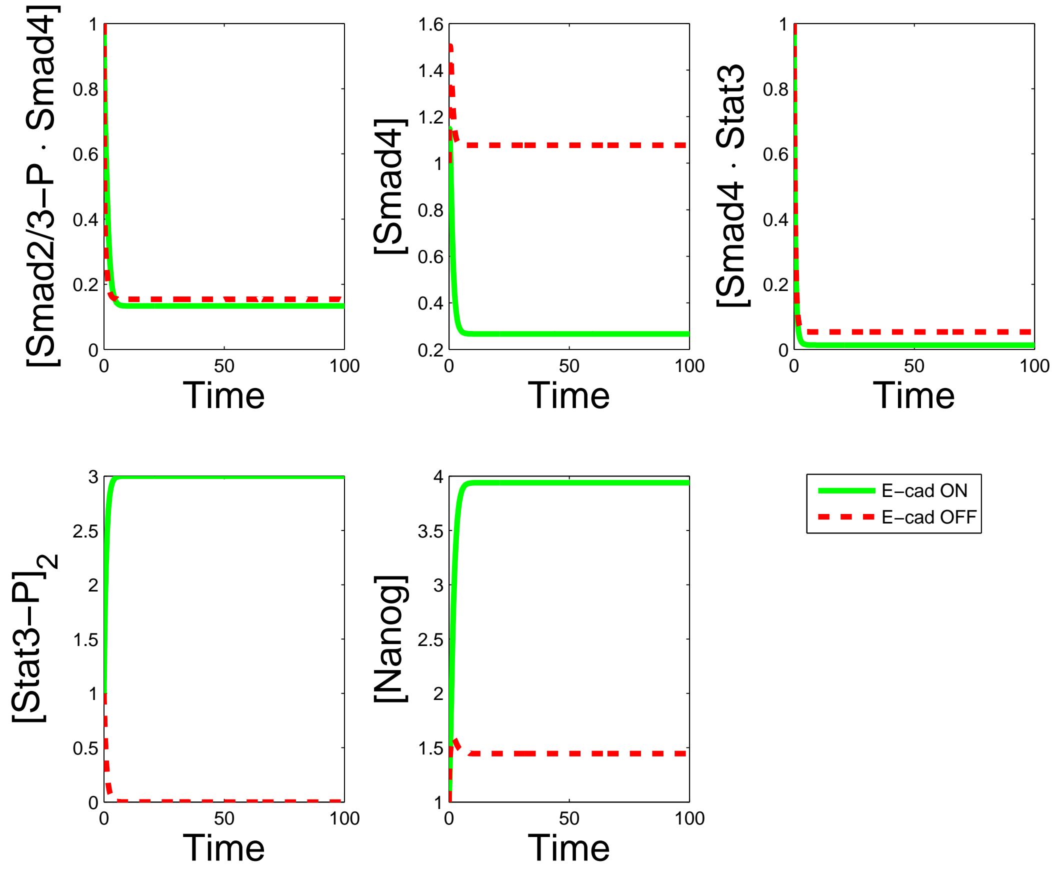


Figure 3: Simulations of the governing system of equations (7)-(12) obtained using the parameter values detailed in Section 2.2.

3 Results and Model validation

Numerical solutions were obtained to equations (7)-(12) using the Runge-Kutta 4th/5th order solver in Matlab (ode45) and the model parameter values detailed in Section 2.2. Model solutions are shown in Figure 3 in the presence and absence of E-cadherin. Here we see that the presence of E-cadherin increases the expression levels of Nanog and Stat3-p_2 whilst all remaining protein and complex concentrations are reduced. These results are in qualitative agreement with the effect of E-cadherin on the expression levels of proteins in the Activin-Nodal an LIF pathways.

In order to check this initial formulation and implementation of our model, we tested the effects of knocking out certain proteins in each of the pathways, to see whether the results of the model agree with experimental findings for how the expression levels of Nanog change

in each case. That is, we checked whether the model reproduces what we initially aimed to include based on the experimental results, as a solid foundation for then testing hypotheses for what is not yet known experimentally. We undertook two different experimental knock outs, eliminating a protein in each of the Activin-Nodal and LIF pathways in turn, to check that each pathway has been formulated correctly.

3.1 Smad2/3/4-P knock-out: testing the Activin-Nodal pathway

First of all, we blocked the production of Smad2/3/4-p from Smad2/3-p and Smad4, in the Activin-Nodal pathway. This corresponds to setting the rate of the forward reaction for the production of Smad2/3/4-p from Smad2/3-p and Smad4 to zero (i.e. setting $\lambda_1 = 0$). This should have no effect on the production of Nanog when E-cadherin is present in our model, since in this instance Nanog is produced by the LIF pathway and not by the Activin-Nodal pathway. However, in the absence of E-cadherin, Nanog production occurs by the Activin-Nodal pathway instead of the LIF pathway. So in the case where no E-cadherin is present, knocking out the production of Smad2/3/4-p should eliminate the production of Nanog. Indeed, the results of numerical simulations show this, since the level of Nanog is unchanged when E-cadherin is present in the model (since Nanog production goes by the LIF pathway); whereas, no Nanog is produced in the knock-out when E-cadherin is absent (as in this case, Nanog is produced by the Activin-Nodal pathway, where a step in this pathway - the production of Smad2/3/4-p - has been blocked). Results shown are for the steady state values of Nanog.

	E-cad ON	E-cad OFF
Wild Type	3.95	1.44
Smad2/3/4-P Knock-out	3.95	0

Table 2: Smad2/3/4-P Knock-out: Nanog expression levels.

3.2 Stat3-p knock-out: testing the LIF pathway

We next looked at targeting the LIF pathway, by blocking the phosphorylation of the Stat3 protein. Experimentally, it has been seen that no Nanog is expressed in mouse embryonic stem cells when Stat3-p is blocked and E-cadherin is present. However, when E-cadherin is removed, Nanog is still expressed when there is no Stat3-p. This is because in the case of no E-cadherin, Nanog is produced by the Activin-Nodal pathway which does not require the phosphorylation of Stat3. However, Stat3-p is required for production of Nanog by the LIF pathway. We made this change in our model by setting the rate of reaction for the phosphorylation of Stat3 to zero: this corresponds to setting $\lambda_3 = 0$, and in our model $\phi_4 = \lambda_3 \cdot S_3$ so we set $\phi_4 = 0$. The results of our model reflect this: there is no Nanog

production in the knock-out when E-cadherin is present; while in the case of no E-cadherin, Nanog is still expressed at the same level for knock-out compared to normal.

	E-cad ON	E-cad OFF
Wild Type	3.95	1.44
Stat3-p Knock-out	0	1.44

Table 3: Stat3-p Knock-out: Nanog expression levels.

A point of discussion is whether the level of Nanog expression should be the same for the no E-cadherin case for normal compared with the case with Stat3-p knocked out. The results in Table 3 were surprising because in our model we have included an interaction between the Activin-Nodal and LIF pathways in the form of Smad4 binding with Stat3 (to produce Smad4·Stat3). This interaction was hypothesised as a way in which Stat3 could reduce the amount of Smad4 available for the Activin-Nodal pathway, as a way towards explaining both why the activin-nodal pathway is less efficient than the LIF pathway for the production of Nanog, and a way in which the two signalling pathways affect each other. We initially thought that removing the phosphorylation of Stat3 would increase the nonphosphorylated Stat3 levels, which would remove more Smad4 from the Activin-Nodal pathway, and hence downregulate Nanog production in the absence of E-cadherin. However, this does not hold because we have assumed that the level of Stat3 is constant in our model. Thus there is no corresponding increase in Smad4·Stat3, and so the level of Smad4 available is not affected by the knock out of Stat3-p and indeed the levels of Nanog expression are unchanged in normal compared to knock-out.

4 Understanding pathway inhibition

4.1 Inhibition of the Activin-Nodal pathway by Stat3-p

As mentioned above, the level of Stat3-p does not affect the production of Nanog in the no E-cadherin case for our current model. However, it is not known in what way E-cadherin exerts its influence on the hierarchy of the signalling pathways; for example, how does the presence of E-cadherin stop the Activin-Nodal pathway from producing Nanog. Including an inhibition of Stat3-p on the production of Nanog by Smad2/3/4-p could test whether the presence of E-cadherin affects Nanog expression levels through Stat3 phosphorylation and whether it is this that affects the hierarchy of the pathways and final Nanog expression levels. In the current model, if we consider the cases of E-cadherin either present or absent and no intermediate states, we see that the inhibition term $\frac{1}{1+\mu S_{3p_2}}(1-\chi)$ has no effect: if $\chi = 1$ (i.e. E-cadherin is present) then this term is clearly 0, while when E-cadherin is absent and $\chi = 0$, no S_{3p_2} is produced. That is, in the following equation, inhibition of

Stat3-p on Nanog currently has no effect:

$$\frac{dN}{dt} = \frac{\lambda_7 S_{234}}{1 + \mu S_{3p_2}}(1 - \chi) + \lambda_5 \chi S_{3p_2} + \lambda_6 \frac{N^2}{N^2 + K^2} - d_3 N.$$

To get around this, we modified the equation for S_{3p_2}, to artificially switch on the production of Stat3-p. Thus, we included this inhibition in our model by changing the equation for the phosphorylation of Stat3 to include the production of Stat3-p when E-cadherin is absent. We did this by changing the χ in front of the production term to be 1 so that this term is non-zero when there is no E-cadherin:

$$\frac{dS_{3p_2}}{dt} = \chi \phi_4 - \lambda_{-3} S_{3p_2}$$

becomes

$$\frac{dS_{3p_2}}{dt} = \phi_4 - \lambda_{-3} S_{3p_2}.$$

Adding the extra inhibition leads to a further downregulation of Nanog in the case when E-cadherin is off, which is what was expected. The qualitative behaviour of the other variables also matches our expectations as shown in Table 4.

	Smad4	Smad2/3/4	Stat3-p$_2$	Smad4 $\cdot$ Stat3	Nanog
E-cad OFF no inhibition	0.15	1.08	0.05	0	1.45
E-cad OFF with inhibition	0.36	1.18	0.06	3	0.9
E-cad ON	0.13	0.27	0.01	3	3.94

Table 4: Inhibition of the Activin-Nodal pathway by Stat3-p.

4.2 Varying E-cadherin expression and determining the mode of inhibition

Our initial modelling focussed on the case of E-cadherin either present or completely absent and did not look at intermediate expression. This was done because experimentally it is usual to consider the normal case and compare it to a subject in which the expression of the gene for E-cadherin has been completely knocked out. However, it is also interesting to consider what happens when there are, for example, low or medium levels of E-cadherin. In fact, later discussions with the experimentalists has suggested that some experimental technique to produce knock-outs are not always as effective as a theoretical total knock-out, so indeed low levels of E-cadherin may be present.

We considered values of χ between 0 and 1 to represent different levels of E-cadherin. We initially expected the relationship between E-cadherin concentration and Nanog levels to be monotonically increasing, since we know that the Activin-Nodal pathway is less efficient at producing Nanog than the LIF pathway. Thus if it were simply a case of switching to the LIF pathway as soon as some E-cadherin were present, we would expect the graph to be increasing. However, the relationship is not monotonically increasing, and rather a slight decrease in Nanog production is seen when E-cadherin is initially introduced in to the system. This is likely due to the inhibition of Stat3-p on Nanog production.

As previously discussed, the inhibition term $\frac{1}{1+\mu S_{3p_2}}(1-\chi)$ has no effect in the cases $\chi = 0$ or $\chi = 1$. However, this term will exhibit an effect for values of χ between 0 and 1. The parameter μ determines the strength of the inhibition of Stat3-p on Nanog production, and it can be seen that varying μ changes the amount that Nanog production decreases when going from no E-cadherin to a little E-cadherin as seen in model simulation results shown in Figure 4 .

The degree of inhibition of Stat3-p on Nanog production is currently unknown. Further experimental work includes measuring Nanog expression with different amounts of E-cadherin present. These results could then be compared with the output of the model to determine the best fit for μ to see the degree of inhibition and give insight into the interaction between the signalling pathways.

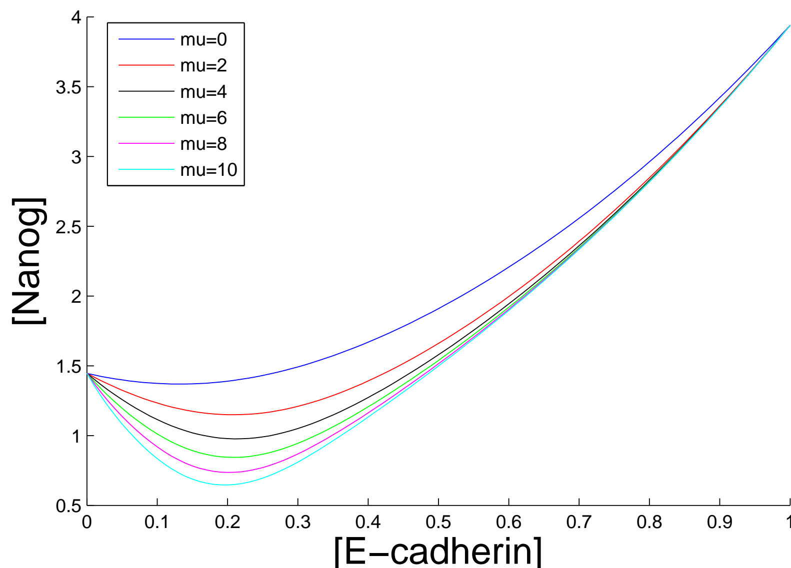


Figure 4: The effect of μ on Nanog expression for varying concentration of E-cadherin.

5 Conclusions

We have developed a nonlinear ODE model of the Activin-Nodal and LIF pathways in embryonic mouse stem cells. The effect of E-cadherin concentration on the expression levels of Nanog have been investigated and our model results have been shown to be in good qualitative agreement with experimental observations. Our work has elucidated the role of Stat3 phosphorylation in affecting the activation of each pathway - increasing unphosphorylated Stat3 does not remove more Smad4 from the Activin-Nodal pathway and thus downregulate Nanog production. The effect of Nanog inhibition for varying concentrations of E-cadherin has been investigated. Both these pieces of work have highlighted the need for further investigation of the of inhibitory effects of Stat3-p on Nanog expression levels, both experimentally and in future modelling work.

References

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