# Distinct Contributions of Tryptophan Residues within the Dimerization Domain to Nanog Function

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## Abstract

The level of the transcription factor Nanog directly determines the efficiency of mouse embryonic stem cell self-renewal. Nanog protein exists as a dimer with the dimerization domain composed of a simple repeat region in which every fifth residue is a tryptophan, the tryptophan repeat (WR). Although WR is necessary to enable Nanog to confer LIF-independent self-renewal, the mechanism of dimerization and the effect of modulating dimerization strength have been unclear. Here we couple mutagenesis with functional and dimerization assays to show that the number of tryptophans within the WR is linked to the strength of homodimerization, Sox2 heterodimerization and self-renewal activity. A reduction in the number of tryptophan residues leads initially to a gradual reduction in activity before a precipitous reduction in activity occurs upon reduction in tryptophan number below eight. Further functional attrition follows subsequent tryptophan number reduction with substitution of all tryptophan residues ablating dimerization and self-renewal function completely. A strong positional influence of tryptophans exists, with residues at the WR termini contributing more to Nanog function, particularly at the N-terminal end. Limited proteolysis demonstrates that a structural core of Nanog encompassing the homeodomain and the tryptophan repeat can support LIF-independent colony formation. These results increase understanding of the molecular interactions occurring between transcription factor subunits at the core of the pluripotency gene regulatory network and will enhance our ability to control pluripotent cell self-renewal and differentiation.
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## Introduction

The processes by which cell fate decisions are made during development are controlled by a temporally and spatially organized hierarchy of transcription factors (TFs) that control gene expression and determine a cell's state. The ability of TFs to mediate these processes relies on their ability to interact with DNA in a sequence specific manner and to interact specifically with other molecules to mediate downstream effects.

Although different TF families recognize DNA and protein partners in different ways, one feature common to many TFs is their ability to form homo- or heterodimers [1]. TF dimerization has a number of functional implications. Bringing two DNA binding regions together can alter or enhance DNA binding specificity. Moreover, dimerization can create contiguous protein surfaces absent from monomers. The transition between monomers and dimers can also be regulated by post-translational modification. For example, Stat protein phosphorylation causes

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dimerization and nuclear translocation [2]. In contrast, estrogen receptor A phosphorylation blocks both dimerization and DNA target binding [3]. For these reasons, the identification of TF dimeric interfaces and the dissection of mechanisms by which dimerization is controlled are important to understand TF function.

In embryonic stem cells (ES cells), a central network of TFs is responsible for the maintenance of ES cell identity. This pluripotency gene regulatory network includes a number of TFs at the core of which is the triumvirate of Nanog, Oct4 and Sox2 [4–9]. Structural information exists for the DNA binding domains for each of these three factors [10–12]. However, although it is known that each of the three proteins can form homo-multimers [13–16], biophysical and structural characterization of the full-length proteins is relatively limited.

The homotypic interaction of Nanog has been characterized in most detail with mouse Nanog shown to exist in solution as a dimer [13,16]. Homodimerization of Nanog is mediated by a region of the protein containing 10 copies of a pentapeptide repeat in which a tryptophan residue is conserved at the same position within each repeat (the tryptophan repeat, or WR) [13,16]. Deletion of the WR from Nanog produces a molecule that cannot confer the defining biochemical property of Nanog, LIF-independent self-renewal [13]. However, the contribution of individual residues to dimerization and cellular function remains unclear. To address these issues, a series of Nanog mutants in the dimerization domain have been constructed and their functional properties investigated.

## Results

### The number of tryptophan residues is a determinant of Nanog activity

A series of Nanog variants were constructed in which one or more tryptophan residues within the WR were mutated to alanine (Fig. 1A and B). The ability of these variants to alter the self-renewal capacity of E14/T ES cells following transfection of constitutive episomal expression vectors was then tested. Initially, individual tryptophan-to-alanine substitutions were assessed. In each case, the ability to confer LIF-independent self-renewal was reduced but this effect was site-specific (Fig. 1D). Replacement of the N-terminal tryptophan (W2–10) reduced the number of undifferentiated ES cells colonies by ~80% compared to wild-type Nanog, whereas replacement of W5 or W10 had a milder effect, reducing activity by ~40%, relative to wild-type Nanog. In the presence of LIF, the effects of W-A substitutions were less severe and in the case of W1–4;6–10, undetectable. Furthermore, the fold-enhancement of self-renewal by LIF was greater for W2–10 than for W1–9. These data indicate that individual tryptophan residues within the WR contribute differentially to LIF-independent ES cell self-renewal, with the tryptophan residue most proximal to the homeodomain having the greatest effect.

The importance of multiple tryptophans was next examined by replacement of two tryptophan residues (Fig. 1E). Combined replacement of W1 with either W10 (W2–9) or W2 (W3–10) reduced self-renewal activity even further, to 5% in the absence of LIF. In contrast, replacement of the two C-terminal tryptophan residues (W1–8) had a more modest effect reducing LIF-independent self-renewal activity to an extent comparable to replacement of the single N-terminal tryptophan (W2–10). However, in contrast to W2–10, W1–8 was compensated less effectively by LIF addition and less effectively than either of the other mutants retaining eight tryptophan residues (Fig. 1E). These data further highlight the important contribution of tryptophan residues at the N-terminus of the WR to ES cell self-renewal activity.

To determine whether the specific sequence of the first repeat within the WR was of greater importance than the actual number of tryptophan residues present in the WR, a mutant was constructed in which the first repeat was deleted and an additional copy of repeat 10 added to the C-terminus of the WR (W2–10/10). This mutant was more similar in function to wild-type Nanog than to either W2–10 or W1–9, suggesting that the position of the first tryptophan of the WR within the overall Nanog structure is of greater importance than the specific sequence of the first repeat within the WR (Fig. 1F).

Alanine replacement of three or more tryptophan residues resulted in further reductions in activity (Fig. 1G). Interestingly, when five tryptophan residues are removed, the differential contribution of the N-terminal tryptophan residues is no longer seen. Instead, an effect of tryptophan adjacency becomes apparent. W1–5 and W6–10 have comparable activities and both exceed the activities of W-odd and W-even, which in the absence of LIF are as negligible as that of W10A. Immunoblot analyses showed that the differential activities of Nanog mutants could not be accounted for by differing protein expression levels (Fig. 1C).

### The number of tryptophan residues determines dimerization efficiency

Nanog dimerization is considered essential for LIF-independent self-renewal [13,16]. To determine whether the loss of function observed when all tryptophan residues are substituted by alanine is reflected in a reduced dimerization ability, co-immunoprecipitation of differentially tagged versions of NanogW10A was performed. This demonstrated that substitution of all W residues in the WR abrogated dimerization capacity (Fig. 2A).

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