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MECHANISMS OF DEVELOPMENT

The PHD domain is required to link *Drosophila* Pygopus to Legless/β-catenin and not to histone H3

*Roman Kessler, George Hausmann, Konrad Basler**

Institut für Molekularbiologie, Universität Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

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ABSTRACT

In *Drosophila* Pygopus (Pygo) and Legless (Lgs)/BCL9 are integral components of the nuclear Wnt/Wg signaling machine. Despite intense research, ideas that account for their mode of action remain speculative. One proposition, based on a recently discovered function of PHD fingers, is that Pygo, through its PHD, may decipher the histone code. We found that human, but not *Drosophila*, Pygo robustly interacts with a histone-H3 peptide methylated at lysine-4. The different binding behavior is due to a single amino acid change that appears unique to *Drosophilidae* Pygo proteins. Rescue experiments with predicted histone binding mutants showed that in *Drosophila* the ability to bind histones is not essential. Further experiments with Pygo–Lgs fusions instead demonstrated that the crucial role of the PHD is to provide an interaction motif to bind Lgs. Our results reveal an interesting evolutionary dichotomy in Pygo structure–function, as well as evidence underpinning the chain of adaptors model.

1. Introduction

Wingless (Wg)/WNT signaling occurs via an evolutionary conserved pathway that plays a fundamental role throughout the life of an organism. At the heart of Wnt target gene regulation lies a nuclear complex that is scaffolded by β-catenin/Armadillo (Arm) (Mosimann et al., 2009). Two regions of Arm/β-catenin are critical for this function: a N-terminal branch and the C-terminal tail (Orsulic and Peifer, 1996). The C-terminal tail serves as an interaction surface for the recruitment of a diverse set of complexes involved in histone modification, transcriptional initiation and elongation (Städeli et al., 2006; Xu and Kimelman, 2007). The function of the N-terminal branch is mediated by the transcriptional potential inherent in the N-terminal Homology Domain (NHD) of Pygopus (Pygo).

The term "chain of adaptors" was coined to describe the recruitment of Pygo (via Legless (Lgs), Arm and Pan/dTCF) to Wg target genes (Städeli and Basler, 2005). Lgs/BCL9 contains two conserved domains that are essential for this function – HD2 (homology domain 2) which mediates the interaction with Arm/β-catenin and HD1 which binds to the PHD of Pygo (Kramps et al., 2002). Reductionist studies in *Drosophila* led to the notion that the sole function of Lgs/BCL9 is to serve as an adaptor between its two partners (Kramps et al., 2002; Städeli and Basler, 2005). A more recent study (Sustmann et al., 2008) ascribed additional activities to domains conserved in mammalian BCL9 orthologs, suggesting that they may have evolved new functions.

The modus operandi of Pygo, in particular the mechanism of action of the NHD, has been fertile ground for speculation (Jessen et al., 2008): the chain of adaptors model in which the Pygo NHD recruits a factor(s) with transcriptional activator function (Hoffmans et al., 2005; Städeli and Basler, 2005; Thompson, 2004); a nuclear retention model in which Pygo functions as a nuclear anchor targeting Arm/β-catenin to

* Corresponding author. Tel.: +41 44 635 3110; fax: +41 44 635 6864.
E-mail address: basler@molbio.uzh.ch (K. Basler).
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the nucleus (Townsley et al., 2004); a refinement of the anchor model in which Pygo functions at the onset of Wnt signaling, or at low signaling levels, to specifically capture Arm at Pan/dTCF target genes (de la Roche and Bienz, 2007).

Another possible mechanism of action for Pygo was raised by the discovery that PHD fingers can act as "code-readers" for localizing protein complexes to gene loci in particular transcriptional states, ranging from inactive (unmethylated H3K4) (Lan et al., 2007) to actively transcribed (H3K4me3) (Li et al., 2006; Pena et al., 2006; Shi et al., 2006; Wysocka et al., 2006). We set out to investigate if this putative interaction could occur and if it is functionally required.

We observed that hPygo, but not dPygo, robustly binds histones. The difference is due to a single amino acid change present in the PHD of all *Drosophilidae* Pygo proteins. In vivo studies demonstrated that in *Drosophila* even if there were an inherent ability to bind histones in the dPygoPHD it is not essential for its function. Experiments with Pygo–Lgs fusions instead showed that the key role of the dPygoPHD is as an adaptor motif to bind Lgs – creating a link in the chain of adaptors. In sum the results highlight a novel evolutionary dichotomy in Pygo structure–function, as well as providing new evidence underpinning the chain of adaptors model.

2. Results

2.1. *hPygo strongly binds to H3K4me2, dPygo does not*

Some PHD fingers, by binding lysine-4 on histone 3 (H3K4), seem to help the proteins containing them to orchestrate gene expression (Mellor, 2006; Ruthenburg et al., 2007). Does the PygoPHD have a similar modus operandi? We found that the PHD of human Pygo2 (hPygo2PHD) bound methylated K4 but not methylated K9 (Fig. 1B). As seen for hPygo1PHD (Fiedler et al., 2008), the hPygo2PHD had a preference for dimethylated K4 (K4me2); in contrast the dNURF301PHD binds K4me3 better (Fig. 1C, (Wysocka et al., 2006)). Unexpectedly we did not see a convincing interaction between the dPygoPHD and H3K4me2/3 (Fig. 1B). Based on the ability of dPygoPHD preparations to bind Lgs we excluded mis-folding as a trivial explanation for the lack of binding (Fig. 3C).

To try to understand why dPygo and hPygo2 behaved differently we examined more closely the PHD sequence. One of the aromatic residues building the so-called K4 cage (Li et al., 2007a; Nakamura et al., 2007) is not conserved in *Drosophila*: hPygo2-W353 vs dPygo-F773 (Fig. 1A). The tryptophan (W), which separates the two pockets for K4 and A1/R2 (Fiedler et al., 2008; Nakamura et al., 2007), is present in PHD fingers that are known to bind methylated histones (Fig. 1A and (Ruthenburg et al., 2007)). Substituting the W for phenylalanine (F) in hPygo2 and dNURF301 abrogated detectable binding (Fig. 1D and Fig. S1). In the converse experiment, replacing the F of dPygo with W (F773W) enabled binding to H3K4me2/3 (Fig. 1D and data not shown).

One interpretation of the above results is that histone binding is not functionally important, at least in *Drosophilidae* where the crucial W is not conserved (Fig. S2). However, an exciting caveat was proposed by Fiedler et al. (2008) – the binding of Pygo to methylated H3K4 (H3K4me) peptides is enhanced by the binding of a peptide corresponding to the HD1 of Lgs.

We also observed that the presence of a HD1 peptide enhanced the Pygo-H3K4 interaction (Fig. 2B). HD1 binding to hPygo2 enhanced the interaction with H3K4me (Fig. 2B). In the presence of HD1, *Drosophila* Pygo also detectably interacted with the H3 peptide; however, the binding was independent of the K4 methylation state (Fig. 2B). This contrasts with Fiedler et al., 2008 who reported no detectable binding by dPygo to the unmethylated peptide.

The HD1 peptide is 34 aa; full-length Lgs and BCL9 are ~1400 aa. The binding of the peptide may therefore not recapitulate the binding of full-length Lgs. We were unable to generate recombinant full-length Lgs/BCL9 to resolve this issue; however, we could generate a truncated version: GST-BCL9 1–300 (Fig. 2A). In contrast to the peptide, BCL9 1–300 abrogated binding of hPygo to H3K4me3 (Fig. 2C). Interestingly, we did not see a strong effect on the binding to H3K4me2. These results suggest Lgs/BCL9 competes with histones for binding to Pygo. However, variables, such as post-translation modifications and other Lgs/BCL9 binding partners (e.g. Arm), may alter this situation – again enabling binding or also inhibiting it. Since these factors cannot easily be tested in a biochemical approach we decided to directly test the importance of histone binding in vivo.

2.2. *Histone binding is not required in vivo*

Previous structural studies had shown that altering residues in the so-called K4 cage abolish the PHD–histone interaction (Fiedler et al., 2008; Li et al., 2006; Pena et al., 2006; Wysocka et al., 2006). Consistent with these reports, mutation of a conserved tyrosine found in the K4 cage effectively abrogated binding to H3K4me2 and H3K4me3 (hPygo2-Y328A, Fig. 3A and data not shown).

A simultaneous effect on Lgs and histone binding would confound interpretation since for such a mutant a loss of function is also predicted by the chain of adaptors model. We therefore wanted to confirm that dPygo-Y748A (corresponds to hPygo2-Y328A) can still bind Lgs. In co-immunoprecipitation and GST-pulldown experiments Y748A was able to bind Lgs (Fig. 3B and C). Conversely, the L789A mutation abolishes Lgs binding but had no effect on histone binding (Fig. 3A–C).

To investigate the biological importance of H3K4 binding we introduced the following mutations: Y748A, A763E, and F773E (and combinations thereof) into a genomic rescue construct of dPygo (Fig. 4A and B). Mutations equivalent to A763E and F773E in hPygo1 abolish histone binding (Fiedler et al., 2008, Fig. S2A shows the corresponding residues in hPygo1, hPygo2 and dPygo).

The constructs were all integrated into the landing site located at cytological position 51D using the attP integration system (Bischof et al., 2007). We then tested if these transgenes could rescue $pygo^{S123.3}/pygo^{EB130}$ or $pygo^{EB130}/pygo^{EB130}$ flies. $pygo^{S123.3}$ has a deletion that only leaves amino acids 1–51 intact (Thompson et al., 2002) and $pygo^{EB130}$ has a deletion starting at amino acid 751, leading to a truncated PHD finger (Kramps et al., 2002).