Chapter 1 Study of Hox protein-protein interactions in living cells using novel fluorescent techniques

Capítulo 1 Estudio de interacciones proteína-proteína Hox en células vivas utilizando técnicas novedosas de fluorescencia

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DOI: 10.35429/H.2022.6.1.1.12

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Abstract

Hox genes are master regulators of development that contain the homeobox, a highly conserved region of 180 base pairs. The homeobox codes for a 60-aminoacid domain called the homeodomain, which interacts with DNA to regulate gene expression with great specificity. How the homeodomain achieves this high level of specificity is one of the great questions in developmental biology. Besides interacting with DNA, the homeodomain also interacts with transcription factors, cofactors, and other proteins to regulate development. These protein-protein interactions are necessary to understand the functions and transcriptional regulation of homeoprotein target genes. In this review, we describe the different techniques used to study Hox protein-protein interactions. These novel fluorescent techniques can be used to verify these interactions in living cells and further analyze them in model organisms to elucidate functional implications of these interactions *in vivo*. As we discover more Hox interacting partners, these techniques will help us determine the essential role of protein-protein interactions within the interactome networks to control cellular functions and morphogenesis and organogenesis *in vivo*.

Protein-protein interactions, BiFC, FRET, Fluorescent techniques, Hox interactome, Cofactors, Transcription regulation, Homeodomain, Development

Resumen

Los genes Hox son reguladores maestros del desarrollo que contienen al “homeobox”, una región altamente conservada de 180 pares de bases. El “homeobox” codifica a una región de 60 aminoácidos llamada homeodominio, la cual interacciona con el ADN para regular la expresión genética con alta especificidad. El mecanismo de cómo el homeodominio logra este alto nivel de especificidad es una de las grandes preguntas en biología del desarrollo. Además de interaccionar con el ADN, el homeodominio también interacciona con factores de transcripción, cofactores, y otras proteínas que regulan el desarrollo. Estas interacciones proteína-proteína son necesarias para entender las funciones y regulación transcripcional de los genes blanco de las homeoproteínas. En esta revisión, describimos las diferentes técnicas utilizadas para estudiar las interacciones proteína-proteína de Hox. Estas novedosas técnicas fluorescentes pueden utilizarse para verificar las interacciones en células vivas y analizarlas en organismos modelo para elucidar las implicaciones funcionales de estas interacciones *in vivo*. Con el descubrimiento de más colaboradores que interaccionan con Hox, estas técnicas nos ayudarán a determinar el papel esencial de las interacciones proteína-proteína dentro de las redes del interactoma que controlan las funciones celulares, la morfogénesis y organogénesis *in vivo*.

Interacciones proteína-proteína, BiFC, FRET, Técnicas fluorescentes, interactoma Hox, Cofactores, Regulación de la transcripción, Homeodominio, Desarrollo

1.1 Introduction

Hox genes are a family of transcription factors (TFs) with a key role as master regulators of development, responsible for giving identity to the segments of the anteroposterior (A-P) axis of bilateral organisms (Lewis *et al*., 1978; Mann and Morata, 2000). All the homeotic genes share a highly conserved region of 180 base pairs (bp) called the homeotic box or "homeobox" (McGinnis *et al*., 1984, Scott & Weiner, 1984) that encodes a 60–amino acid DNA-binding domain known as the homeodomain (HD) (Gehring, 1987). The HD’s tridimensional structure consisting of three alpha-helixes allows it to bind to DNA, specifically through helix III—the recognition helix—that contains short regions rich in A-T present in the major groove of DNA (Svingen & Tonissen, 2006, Mann *et al*., 2009).

However, considering that the HD of all homeoproteins is highly similar and that the DNA regions to which they bind are also present multiple times throughout the entire genome. In addition, some Hox proteins bind cooperatively to chromatin (Arunachalam, *et al*., 2022; Latham & Zhang, 2022). It is therefore difficult to imagine how each of the homeoproteins is capable of specifically regulating target genes for the development of the segments along the A-P axis of the different organisms. This question is known as the “Hox paradox” (Prince *et al*., 2008; Hueber & Lohman, 2008).

Many transcriptional factors that interact with homeoproteins have been described in the complete map of protein interactions called the "Hox interactome" (Baëza *et al*., 2015). Protein-protein (PPI) interactions became relevant when it was determined that Extradenticle (Exd) can interact with homeoproteins through the YPWM motif, improving the specificity of DNA binding site compared to its monomeric form (Shanmugam *et al*.,1997; Joshi *et al*.,2007). Exd dimeric interaction with homeoproteins like Abdominal A (abdA), Ultrabithorax (Ubx), Sex combs reduced (Scr), and Antennapedia (Antp) (Passner *et al*.,1999; Joshi *et al*., 2007; Papadopoulos et al., 2011) increase the specificity of homeoproteins. Homothorax (Hth), another Hox cofactor that establishes interaction with homeoproteins, also interacts with Exd to allow active nuclear export (Rieckhof *et al*., 1997; Ryoo *et al*.,1999). Both cofactors interact with homeoproteins, forming trimeric Hox-Exd-Hth complexes that improve specificity, allowing the activation or repression of different genes. For example, Ubx-Exd-Hth and AbdA-Exd-Hth trimers can activate or repress transcription depending on the cis-regulatory module (CRM) to which it binds. (Merabet *et al*.,2007; Delker *et al*.,2019 Zandvakili *et al*., 2019). Although these trimeric complexes between homeoproteins with cofactors increase specificity, it is not enough to explain the transcriptional regulation given by homeoproteins.

To specify cellular identity, homeoproteins interact with cofactors such as Exd, but also with general transcription factors (GTF) that confer specificity. The first link of functional contact with a GTF found in *Drosophila* was between Antp and BIP2 (TAFII155, TAF3), a member of the TFIID complex (Prince *et al*., 2008). Other homeoprotein interactions include components of Pol II machinery, Med19 from the Mediator (MED) complex, and the transcription-pausing factor M1BP and TFIIEβ (Boube *et al*., 2014; Zouaz *et al*., 2019; Baëza *et al*., 2015). These interactions demonstrate Hox-driven transcription, but the molecular mechanisms behind transcriptional regulation remain elusive.

1.2 Analysis of protein-protein interactions

Protein-protein interactions (PPIs) are necessary to understand the functions and transcriptional regulation of homeoprotein target genes. Several techniques are used to study protein-protein interactions *in vitro*; of these, Co-IP, pull-down assays, affinity purification coupled to mass spectrometry (TAP-MS), and yeast two-hybrid have been used to study the Hox interactome and are described below.

In Co-IP, the target protein is recognized by a specific antibody that has been immobilized to a support, which is then used to co-precipitate the antibody-protein complex from the cell lysate. The complexes are detected and identified by western blot analysis or mass spectrometry (Free *et al*., 2009). Interactomes of the Hox proteins Abd-A, Antp, Scr and Ubx have been validated by Co-IP experiments (Baëza *et al*., 2015). Ubx partners have also been identified from nuclear extracts of embryos (Carnesecchi *et al*., 2020).

In pull-down assays, the detection system consists of a glutathione S-transferase (GST)-, polyHis- or streptavidin-tagged protein that is captured by agarose beads coated with the tag-specific ligand. The immobilized fusion-tagged protein captures its putative interacting partner from a cell lysate; the complexes are selectively eluted for in-gel or western blot analysis (Brymora *et al*., 2004). A great number of Hox PPIs have been identified by pull-down assays including cofactors, GTFs and other Hox proteins (Emili *et al*., 1994; Zhu and Kuziora 1996; Rieckhoff *et al*., 1997).

The TAP-MS assays require two tags instead of one in a sequential manner; typically, a combination of FLAG and HA peptides are used as affinity tags (Rigaut *et al*., 1999; Nakatani and Ogryzko 2003). In the first affinity step, the TAP-tagged protein is expressed in cells for association with its endogenous partners. In the second step, the complexes formed are purified by interaction with the corresponding tag ligand using immunoglobulins anchored on agarose beads. Once recovered, protein complexes can be processed for direct analysis by mass spectrometry (MS) or separated by SDS-PAGE for reducing sample complexity before MS analysis (Dunham *et al*., 2012; Adelmant *et al*., 2019). This approach has been used to capture lineage-specific Ubx partners at the subcellular level (Carnesecchi *et al*., 2020). Also, the interactome of the Hox cofactor Meis1a was analyzed using TAP-MS in absence or presence of its regulator Prep1 to gain insight into its oncogenic activity (Dardaei *et al*., 2014).

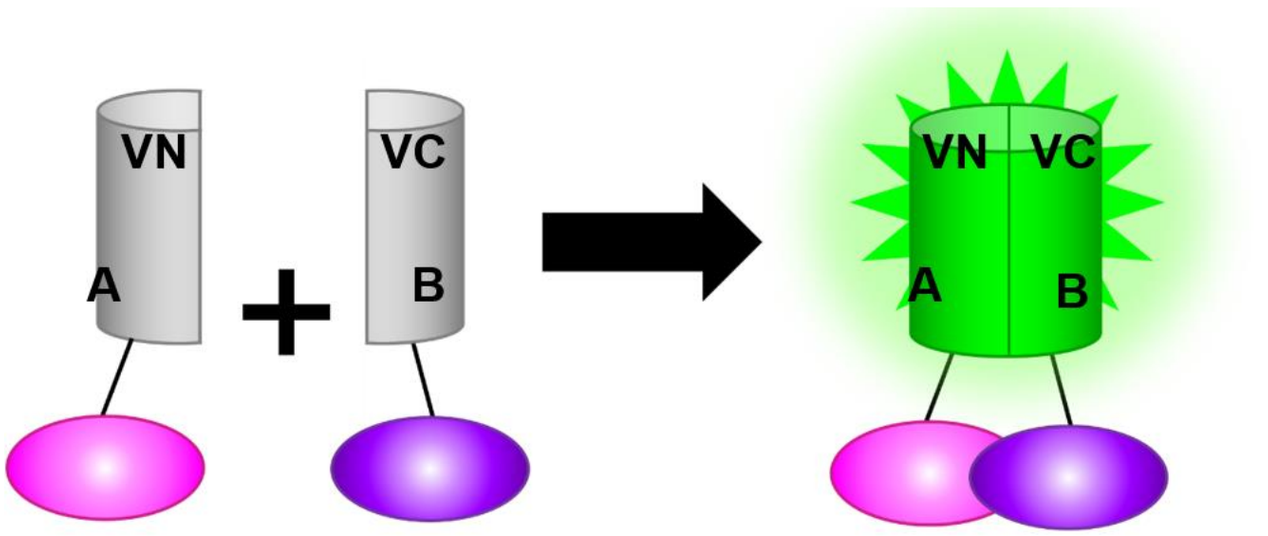
The yeast two-hybrid (Y2H) assay allows the identification of PPIs using the yeast Gal4 transcription factor. The DNA-binding domain and activation domain of Gal4 are fused to two proteins of interest. If both proteins interact, Gal4 transcription factor becomes functional, activating the expression of reporter genes under the control of the UAS (*GAL* promoter). This assay is used to confirm the interaction between two proteins of interest and discover novel PPIs (Fields and Song 1989). Y2H screenings identified Ubx interacting proteins like TFs and cell signaling regulators (Bondos et al., 2006). Another example is the identification of the homeodomain-interacting protein kinases as a novel family of Hox co-repressors that differentially interact with homeoproteins in living cells (Kim et al., 1998).

Overall, these *in vitro* techniques are very popular for PPI detection and could be used in large-screening approaches. However, there are some drawbacks that must be considered when choosing the most appropriate technique, because these methods rely on indirect detection of the PPI or require disruption of the cells. Some PPIs are transient or weak, or require a specific cellular environment, giving false positive or false negative results; in other words, only strong and stable PPIs can be detected. Also, since these methodologies require lysis of the cell, the compartment in which the PPI occurs cannot be determined.

Fluorescent labeling techniques can be used to detect protein-protein interactions in live conditions and visualize the location where they occur within the cell, allowing us to better understand protein function *in vivo*. For example, fluorescent fusion proteins have been used to visualize gene expression of the Hox locus at the subnuclear level in *Drosophila* imaginal discs (Delker *et al.*, 2022). Some techniques used to analyze PPIs are fluorescence anisotropy, fluorescence correlation spectroscopy (FCS), fluorescence lifetime image microscopy (FLIM) and photonic crystal (PC) biosensors. The most commonly used techniques to study PPIs are Bimolecular fluorescent complementation (BIFC), Förster resonance energy transfer (FRET), competitive BiFC, and the combined BiFC-FRET.

Bimolecular fluorescence complementation (BiFC) relies on a visible fluorescent signal. This assay is based on complementation between two fragments of a fluorescent protein that are each fused to a protein of interest. The interaction between these proteins brings the fragments together and reconstitutes the fluorescence, indicating protein-protein interaction (Figure 1). The first fluorescent protein used in complementation assays was the green fluorescent protein (GFP); now there are various GFP-derivatives such as the YFP, Venus or Cerulean (Hu *et al.*, 2002; Hu and Kerppola, 2003; Shyu *et al.*, 2006), red fluorescent variants like mRFP1 (Jach *et al.*, 2006) and lately the near infrared fluorescent protein iRFP (Chen *et al.*, 2015). Venus has been used to determine Antp-TFIIEβ interaction, showing a direct physical interaction of TFIIEβ with two aminoacidic positions on helix 2 of the Antp HD that is required for the ectopic function of Antp in thorax and antenna-to-tarsus transformations. Antp-TFIIEβ interaction showed the important relationship between Hox proteins and the general transcription machinery for transcriptional regulation (Altamirano-Torres *et al*., 2018).

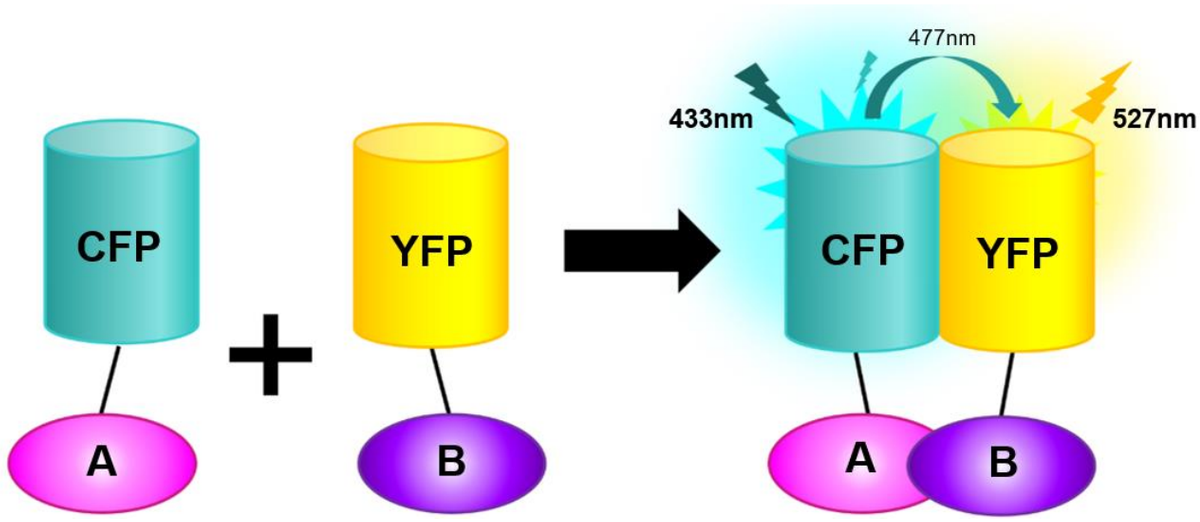
Figure 1 Schematic representation of BIFC. Venus fragments (VN AND VC) are fused to A and B proteins of interest. The interaction of A and B proteins brings the Venus fragments together and enables Venus fluorescence, indicating an interaction between A and B



BiFC has been used successfully in yeast (Sung *et al*., 2013), plants (Lee *et al*., 2012), mammalian cells (Lee *et al*., 2011) and insects like *Drosophila melanogaster* (Plaza *et al*., 2008) allowing us to study PPIs in a tissue- and developmental stage–specific manner *in vivo*. Its main advantage is that the complementation between the two fragments causes covalent junctions, leading to the stabilization of the protein complex. Although it is not possible to monitor the temporal dynamics of PPIs, BiFC allows detection of transient and weak interactions that occur simultaneously, combining peptides of different fluorescent proteins (multicolor BiFC) (Bischof *et al*., 2018). This assay was applied to detect interactions in living embryos in *Drosophila* (Hudry *et al*., 2011) and to create a BiFC library consisting of a collection UAS-ORF fly lines that allows the simultaneous detection of PPIs in living embryos. This multicolor BiFC library is updated constantly and aims to cover all *Drosophila* TFs in the near future (Bischof *et al*., 2013).

Förster resonance energy transfer (FRET) is another method that enables the detection of interactions *in vivo.* FRET can measure the proximity between two fluorophores that are fused to interest proteins, upon excitation. The energy transfer between a donor and an acceptor molecule should occur within a small distance (less than 10 nm) to validate the close proximity between interest proteins and hence the interaction between them (Shyu, *et al.*, 2006, Truong & Ikura, 2001, Parsons *et al.*, 2004, Kenworthy, 2001). The efficiency of the energy transfer refers to donor excitation that results in energy transfer to the acceptor, thus the donor emission decreases whereas the acceptor emission increases, indicating PPI (Figure 2). A variation of FRET is BRET (bioluminescence resonance energy transfer), where a light-emitting enzyme (luciferase) is used as a donor, and a fluorescent protein is used as an acceptor (Harikumar *et al.*, 2017).

Figure 2 Schematic representation of FRET. Donor (CFP) is fused to protein A and acceptor (YFP), to protein B. When the donor is excited, energy is transferred to the acceptor, decreasing the donor's fluorescence, and indicating A and B interaction

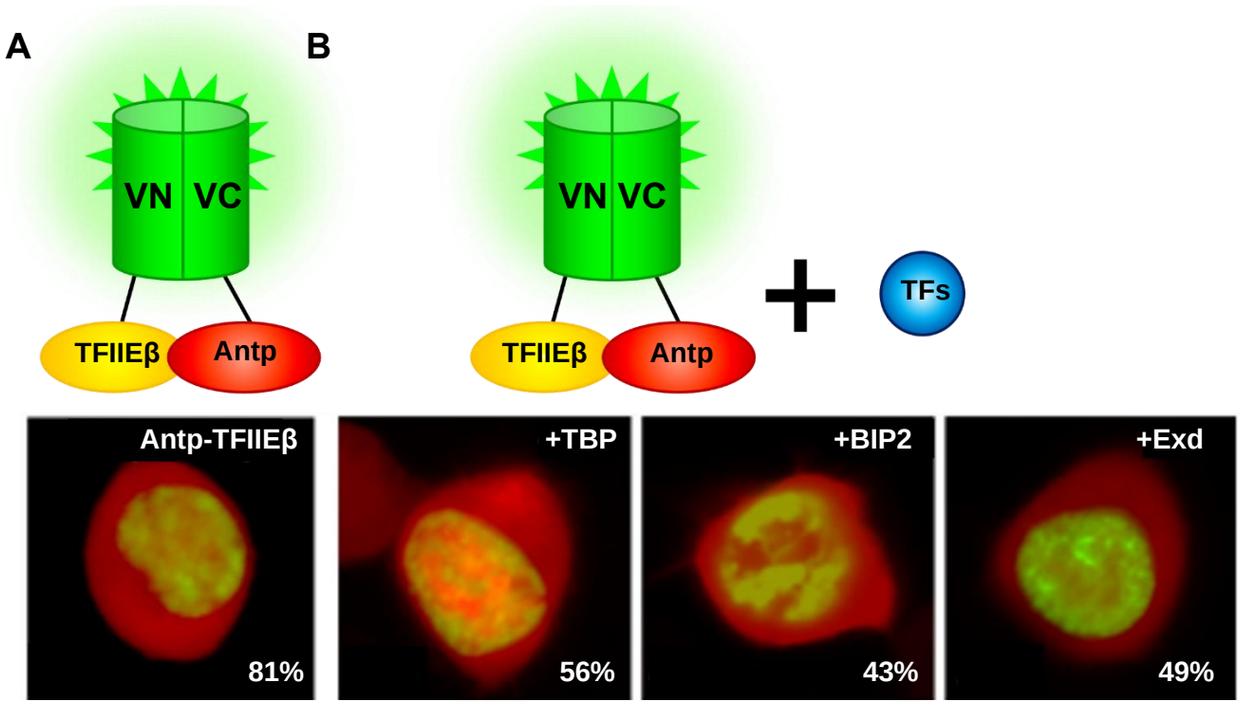


In FRET, the most commonly used fluorescent proteins are CFP and YFP, although the YFP mutant Venus and the CFP mutant Cerulean have been used successfully, showing better properties for live-cell imaging. These techniques require high levels of protein expression as well as specific interfaces to reliably interpret the few emitted signals and therefore cannot be used for large-scale applications.

BiFC and FRET assays allow the detection of dimeric interactions, and multicolor BiFC can detect several dimers simultaneously; detecting trimeric complexes, however, has proved to be a challenging task. In 2004, Galperin et al., developed a three chromophore–based FRET system that can measure the signals of three donor-acceptor pairs, such as CFP-YFP, CFP-mRFP and YFP-mRFP *in vitro* and *in vivo*, and can be used to visualize trimeric complexes in cells, but required sophisticated optical setup and data process.

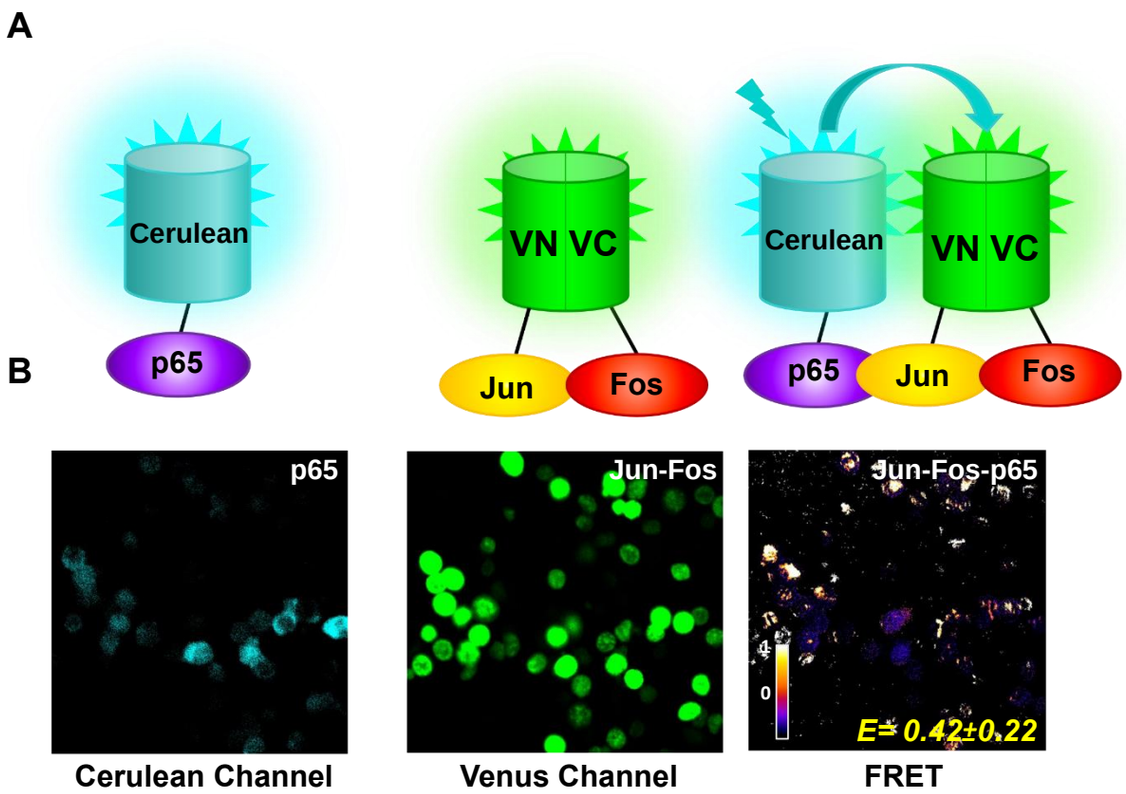
One approach to indirectly detect possible candidates for trimeric interactions are competition experiments. Here, a “cold” competitive partner (not fused to a fluorescent protein fragment) is co-expressed with proteins fused to the amino and carboxyl ends of fluorophore, expecting a decrease in dimeric interaction (Baëza *et al*., 2015). An example is shown in Figure 3, where Antp and TFIIEβ interact in HEK293 cells, giving an interaction of 81%. When the “cold” proteins (TBP, BIP2 and EXD) were co-transfected with the Antp/TFIIEβ complex the interaction decreased, indicating the possible formation of trimeric interactions with these transcriptional factors.

Figure 3 Antp-TFIIEβ interaction is affected by TBP, BIP2 and Exd in competitive BiFC assays. The co-expression of Antp and TFIIEβ with TBP, BIP2 and Exd. A) Green fluorescence indicates the protein-protein interaction of Antp with TFIIEβ in 81% of the transfected cells. B) The addition of TFs to Antp-TFIIEβ decreased the interaction to 56% in TBP, 43% in BIP2 and 49% in Exd. pCAG-mCherry was used as an internal control for transfection



A better option to detect trimeric complexes directly is the combination of BiFC and FRET (BiFC-FRET) using Venus and Cerulean fluorescent proteins for visualization of trimeric complexes in living cells (Shyu *et al.,* 2008), avoiding the use of three chromophores. BiFC allows reconstitution of a fluorescent protein (with proteins of interest fused to the fragments) and can be used as a donor, plus Cerulean fused to another protein used as an acceptor. An example of BiFC-FRET being used to detect a trimeric interaction between p65-Jun-Fos is observed in Figure 4.

Figure 4 P65-Jun-Fos trimeric interaction by BiFC-FRET. (A) Schematic representation of p65 fused to Cerulean (Cerulean Channel), BiFC by Jun-Fos interaction (Venus channel) and energy transfer due to p65-Jun-Fos trimeric complex (FRET). Modified from Jiménez-Mejía *et al.*, 2018



In summary, *in vivo* approaches enable detection of PPIs in their normal environment without disrupting the cell, and allowing their visualization and localization, all important aspects to determine their function during development. Moreover, these techniques can determine key interacting domains that could be useful, not only to elucidate function, but to find ways to disrupt interactions involved in pathologies such as cancer. These next-generation therapeutic approaches have been successfully used to disrupt the interaction between HOX proteins and their co-factors in neoplasms such as glioblastoma multiforme, leukemia and other neoplasms (Morgan *et al*., 2017; Arunachalam, *et al*., 2022).

1.3 Acknowledgements

We thank Consejo Nacional de Ciencia y Tecnologia (CONACyT) and Programa de Apoyo a la Investigación en Ciencia y Tecnología (PAICyT) for financial support.

1.4 Funding

The present work was financed by CONACYT, project number CF-2019-2280 and PAICyT project number CN1558-21. AVP received the scholarship number 717425 and CHB, scholarship number 790953.

1.5 Conclusion

Proteins control basically all biological systems; they rarely act alone and most of them interact with others to perform their biological activity in the cell. Protein-protein interactions are the physical contacts with molecular docking between proteins that allow them to achieve their biological functions both at cellular and organism level, integrating the so-called interactome. In this review we analyze how methods such as co-immunoprecipitation, pull-down assays, BiFC, FRET and BIFC can be used to analyze the PPIs of Hox proteins in order to understand the Hox interactome. These novel fluorescent techniques present the advantage to verify these interactions in living cells and further analyze them in whole in model organisms to elucidated functional implications of these interactions *in vivo.*

Although we have made significant progress in the study of Hox interacting partners to understand how multimeric complexes between homeoproteins and cofactors increase the specificity of transcriptional regulation to specify cellular identity, we still possess a limited understanding of how Hox PPIs control cellular functions and morphogenesis and organogenesis *in vivo*.

Moreover, data published in recent years with efficient large-scale technologies that measure proteome-wide physical connections produced a large collection of PPI databases that are available in public repositories. All these PPIs complexes should be analyzed in an adequate biological context to determine their essential role to build and analyze interactome networks.

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