ABSTRACT

The growth of a single cell into multicellular organism is regulated by a few key signaling pathways. The ability of these basic signaling pathways, to regulate bewildering array of cellular and developmental processes, is achieved by a massive crosstalk that occur between them and by context specific interactions with other proteins. Further, these pathways are strictly regulated at multiple steps to fine tune the signaling outcome in a specific cellular context. Evolutionarily conserved Notch pathway is one such widely used intercellular communication systems that regulate proper growth and development of multicellular organisms. The Notch mutant was isolated more than a century ago as a dominant X-linked mutation that exhibits notches in wing margin phenotype in *Drosophila* melanogaster and hence derived its name 'Notch' (Mohr 1919, Morgan and Bridges, 1916). Several genetic, molecular, biochemical and bioinformatic approaches have been used to unravel the components of Notch circuit. Pleiotropic Notch functions in dose dependent manner to regulate different developmental processes such as cell fate determination, differentiation, proliferation, apoptosis, stem cell maintenance etc. One of the most unique properties of Notch signaling is that it is highly pleiotropic and the signaling output is depends on different developmental and cellular contexts. Decades after its discovery, Notch gene was cloned in *Drosophila* and molecular characterization revealed that the gene encodes a transmembrane receptor. The Notch receptor is translated as a 300 kDa polypeptide and during its maturation in *trans*-Golgi network, full length protein is proteolytically cleaved by Furin-like convertases (S1 cleavage) which give rise to a 180 kDa N-terminal extracellular subunit (NEC) and a 120 kDa C-terminal transmembrane intracellular subunit (NTM) (Blaumueller et al. 1997). This heterodimeric Notch receptor is translocated to the cell membrane where it interacts with ligands of the DSL family (Delta and Serrate/Jagged in *Drosophila* and mammals and LAG-2 in *C. elegans*) from the neighboring cell. Binding of ligands to extracellular domain leads to second proteolytic cleavage (S2) by ADAM family of metalloproteases (Brou et al. 2000). This is followed by an intramembrane cleavage (S3) by γ-secretase complex (Presenilin, Nicastrin, PEN-2 and APH-1). Notch

intracellular domain (Notch-ICD) is released from the membrane (Brou et al. 2000, De strooper et al. 1999, Struhl and Greenwald 1999, Ye et al. 1999), and translocates to the nucleus. In the nucleus, Notch-ICD directly binds to CSL transcription factor (*Drosophila* Suppressor of Hairless/*C. elegans* LAG-1/RBP-jk or CBF1 of Mammals) and recruits transcriptional co-activators like Mastermind (Mam) in *Drosophila*/ Mastermind-Like (MAML) in Mammals. This ternary complex also recruits histone acetylase CBP/p300 and SKIP leading to activation of Notch target genes. This association converts CSL transcriptional repressor to transcriptional activator and turns on the transcription of downstream target genes (Struhl and Adachi 1998, Kopan et al. 1994). Most classical target genes of Notch signaling belong to basic helix-loop-helix families of transcription factor, *Enhancer of Split* [*E(spl*)] in *Drosophila*, *hairy/ enhancer of split* (HES) and *Hrt* (Hes-related) or *hairy/enhancer of split-related with YRPW motif* (Hey, also called HESR) in mammals. These bHLH transcription factors in turn repress *achaete-scute complex* (As-C) proneural genes (Campos-Ortega 1993, Fortini and Artavanis-Tsakonas 1994, Wu et al. 2000). Depending upon the cellular context *wg*, *cut*, *string*, *c-myc*, *cyclinD* etc are also Notch target genes (reviewed in Bray and Bernard 2010). The Notch pathway is regulated at multiple levels include patterns of receptor and ligand expression, Notch-ligand interactions, trafficking of the receptor and ligands, and covalent modifications including glycosylation, phosphorylation, and ubiquitination of the receptor (reviewed in Baron et al. 2002, Andersson et al. 2011). Studies on loss of Notch function in *Drosophila* embryo revealed that Notch is a 'Neurogenic gene' because it produces remarkable excess of neurons at the expense of the epidermis (Poulson, 1937; Lehmann et al., 1983). Other than neurogenesis, Notch plays an indispensible role in development of almost every organ and tissue. As a result, any aberration in Notch function leads to many diseases in human including cancer. In recent decades, accumulating evidence has linked alterations in the Notch pathway to tumorigenesis. Further research has established a well-defined role for Notch signaling in haematological malignancies, and recent studies have showed evidences for the significant role of the pathway in solid tumors as well (Aster et al., 2000; Girard et al., 1996; Allman et al., 2001; Gallahan and Callahan, 1987; Dievart et al., 1999; Weijzen et al., 2002; Zagouras et al., 1995, Ranganathan et al., 2011; Radtke and Raj, 2003). There have been several reports of

integration of Notch signaling with other signaling pathways that might attribute to its pleiotropic functions. Notch interacts with other proteins in a combinatorial and context-dependent manner to produce the wide and diverse range of downstream events required for development, homeostasis, and disease. This intricate regulatory mechanism accounts for its versatility to influence different aspects of development.

In an effort to identify novel components involved in Notch signaling and its regulation, yeast two-hybrid screens using different portions of the intracellular domain of Notch receptor as baits were carried out in our laboratory and a chromatin binding protein, Hat-trick, was identified as an interactor of Notch-ICD. In the present study, functional characterization of Hat-trick, as a novel interacting partner of Notch-ICD, was carried out.

Hat-trick is a putative DNA binding protein that harbors ARID, Chromo-domain and Tudor-domain. These domains are present in proteins which are involved in DNA binding and chromatin modeling (Akhtar et al., 2000; Bouazoune et al., 2002; Brehm et al., 2000; Kim et al., 2006; Iwahara and Clubb, 1999; Iwahara et al., 2002; Herrscher et al., 1995). Through bioinformatics analysis Hat-trick is presumed to be a chromatin modeling protein and its functional characterization is not yet complete. It has derived its name from its putative role in heterochromatinization and its influence on TDP-43 mediated toxicity (Sreedharan et al., 2015). The gene *hat-trick* encodes two annotated transcript variants which could be translated into two polypeptides of size 186 kDa and 259 kDa, respectively. Because of presence of such important chromatin remodeling and DNA binding domains, it was fascinating to elucidate the pleiotropic functions of Hat-trick during development and in regulation of Notch signaling.

During initiation of the project, Hat-trick was only represented by annotated symbol CG34422. Available bioinformatic data revealed that this protein is a putative chromatin modeling protein and hence can regulate a wide array of genes during development. In the same hunch, we began the functional characterization of *hat-trick*, its expression analysis, and observation of its loss-of-function and gain-of-function effects. Protein analysis revealed a predominant nuclear expression in different embryonic, larval and adult tissues through immunostaining. Expression

analysis of *hat-trick* transcripts confirmed its homogenous expression in different *Drosophila* tissues. Fascinatingly, a predominant nuclear expression of Hat-trick protein was depicted in oocyte nucleus during various stages of oogenesis. A very few number of proteins are known to show a similar expression pattern. This interesting expression pattern prompted us to explore the functions of Hat-trick during oogenesis. We report for the first time that *hat-trick* has profound multifaceted role during oogenesis in *Drosophila*. Our present analyses reveal that *hat-trick* is required for cytoblast proliferation, double-strand break repair, oocyte determination, cyst encapsulation, nurse cell endoreplication and their migration, and Gurken mediated dorso-ventral patterning during oogenesis. Our results clearly demonstrate that *hat-trick* has an indispensible role in early oogenesis.

The Notch transactivation mechanism resembles other inducible enhancers system, as Notch target genes are actively repressed in the absence of signaling by the association of CSL proteins with histone deacetylases and CtBP, SMRT, or CIR corepressors (Dou et al. 1994; Kao et al. 1998; Hsieh et al.1999; Morel et al. 2001) and binding of Notch-ICD with CSL replaces co-repressors with co-activators like Mastermind (MAM; Smoller et al. 1990; Xu et al. 1990), SKIP (Zhou et al. 2000), histone acetyltransferases PCAF/GCN-5 (Kurooka and Honjo 2000) and CBP/p300 (Oswald et al. 2001). In past decades several proteomic approaches have expanded the number of proteins that are involved in Notch transcription complexes and their targets. Despite the ample of information on the regulatory cascade of Notch signaling pathway, still the specific mechanisms behind the contexts dependent functions of Notch remain largely opaque. The context specific activation of different Notch targets depends on several factors including proteins that are components of activation complex. This is not yet completely understood that how the transcriptional activation complex is regulated depending on different contextual cues. In any given cellular context, only a subset of target genes is transcribed. Combinatorial interactions between Notch transcription complexes and tissue-specific transcription factor activators bound to target gene cis-regulatory elements are an effective mechanism to selectively activate transcription. Since, Hat-trick is also presumed to be a DNA-binding protein, therefore it was very intriguing to decipher the role of a chromatin modeling protein, *hat-trick* in regulation of Notch signaling. We characterized the

functional significance of Notch and Hat-trick interaction. It was observed that Hat-trick physically associates with Notch-ICD, interacts genetically with Notch pathway components. We have carried out experiments to propose that Hat-trick is a part of Notch-Su(H) activation complex. Using molecular and genetic analyses, we found that Hat-trick acts as positive regulator Notch signaling. L-o-f and g-o-f studies revealed that Hat-trick is a positive regulator of Notch signaling. Hat-trick cooperates with Notch-ICD and Su(H) to form activation complex at the regulatory sequences of Notch downstream targets, *Enhancer of Split* [*E(spl)*] complex genes and consequently results in their expression. We also observed that a synergistic relationship occurs between Notch and Hat-trick which results in JNK signal activation, that promote tissue disorganization and neoplastic transformation in Notch mediated tumorigenesis.