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MAPK-dependent phosphorylation modulates the activity of Suppressor of Hairless in *Drosophila*

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ABSTRACT

Cell differentiation strictly depends on the epidermal growth factor receptor (EGFR)- and Notch-signalling pathways, which are closely intertwined. Here we address the molecular cross talk at the level of Suppressor of Hairless [Su(H)]. The *Drosophila* transcription factor Su(H) mediates Notch signalling at the DNA level: in the presence of signalling input Su(H) assembles an activator complex on Notch target genes and a repressor complex in its absence. Su(H) contains a highly conserved mitogen activated protein kinase (MAPK) target sequence. Here we provide evidence that Su(H) is phosphorylated in response to MAPK activity. Mutation of the Su(H) MAPK-site modulated the Notch signalling output: whereas a phospho-deficient $Su(H)^{MAPK\text{-}ko}$ isoform provoked a stronger Notch signalling activity, a phospho-mimetic $Su(H)^{MAPK\text{-}ac}$ mutant resulted in its attenuation. In vivo assays in *Drosophila* cell culture as well as in flies support the idea that Su(H) phosphorylation affects the dynamics of repressor or activator complex formation or the transition from the one into the other complex. In summary, the phosphorylation of Su(H) attenuates Notch signalling in vivo in several developmental settings. Consequently, a decrease of EGFR signal causes an increase of Notch signalling intensity. Hence, the antagonistic relationship between EGFR- and Notch-signalling pathways may involve a direct modification of Su(H) by MAPK in several developmental contexts of fly development. The high sequence conservation of the MAPK target site in the mammalian Su(H) homologues supports the idea that EGFR signalling impacts on Notch activity in a similar way in humans as well.

1. Introduction

The spatiotemporal development of multicellular organisms is coordinated by a small number of signalling cascades, which are reiteratively used from the earliest up to the final developmental decisions. These signalling pathways are well conserved in evolution. Moreover, they form a complex signalling network to allow coordination of developmental processes (for a review, see [1,2]). Deciphering the composition of this network is key to understanding organismal development. Mounting evidence indicates that signalling pathways are extremely versatile in inducing specific responses to different developmental contexts largely due to their ability to integrate various signals at different nodes of their transduction cascade. The highly conserved epidermal growth factor receptor (EGFR) and Notch signalling pathways are both prime examples. They are engaged in various differentiation processes in invertebrates as well as in vertebrates (for review: [2–4]). The transmembrane receptor Notch receives signals from the neighbouring cell through binding of one of its membrane tethered ligands, triggering several cleavage events in the Notch receptor and resulting in the release of the intracellular Notch domain (ICN) from the membrane. ICN shuttles to the nucleus to set up a transcriptional activator complex with the DNA binding transcription factor CSL (CBF1 in human; Suppressor of Hairless [Su(H)] in *Drosophila*; and Lin12 and Glp-1 [LAG1] in *Caenorhabditis elegans*) plus additional transcriptional activators such as Mastermind [5,6]. CSL consists of three structural domains: the N-terminal domain NTD and the beta-trefoil domain BTD both bind to DNA, whereas the BTD and the C-terminal domain CTD interact with ICN [7]. In the absence of signalling, CSL recruits a transcriptional repressor complex to silence Notch target gene transcription [8]. In *Drosophila*, Hairless (H) is central to repressor complex formation: binding to the CTD of Su(H), Hairless mediates transcriptional silencing of Notch target genes by recruiting the global corepressors Groucho (Gro) and C-terminal binding protein (CtBP) [9]. In addition, epigenetic regulators like the histone demethylase KDM5A/Lid were shown to be directly associated with Su(H), thereby contributing to the delicate regulation of Notch signalling activity [10,11]. In contrast to Notch

Abbreviations: ANK, ankyrin repeats domain of Notch; Bx, Beadex; CSL, acronym of CBF1 (human C-promoter binding factor 1) Su(H) (Suppressor of Hairless, *Drosophila melanogaster*) and Lag-1 (lin-12 and Glp-1 phenotype, *Caenorhabditis elegans*); EGFR, epidermal growth factor receptor; en, engrailed; Eq, equator; GFP, green fluorescent protein; MAPK, mitogen activated protein kinase; QE, quadrant enhancer; RTK, receptor tyrosine kinase; Su(H), Suppressor of Hairless; UAS, upstream activating sequences.

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signalling, the activation of the EGFR induces a relay of different kinases leading to the phosphorylation and activation of the Mitogen-activated protein kinase (MAPK), which in turn enters the nucleus to post-transcriptionally modify the activity of specific target proteins, notably transcriptional regulators (for a review, see [3,12]). Two of the first identified substrates of MAPK in *Drosophila* were the transcriptional regulators Pointed and Yan, whose activity is either stimulated (Pointed) or alleviated (Yan) upon phosphorylation [12]. Several studies in the past years provided us with numerous further target transcription factors, indicating that MAPK is an important junction in the cross talk of EGFR signalling with other signalling pathways. Therefore, it is not astonishing that MAPK signalling is molecularly linked to such diverse processes as cell proliferation and apoptosis, morphogenesis and differentiation [13–16]. Moreover, not only transcriptional activators but also repressors were shown to succumb to MAPK-dependent phosphorylation. One example is Gro, whose repressor capacity has lately emerged to be impeded by MAPK-dependent phosphorylation [17]. Gro is implicated as corepressor in various signalling cascades, including the Notch pathway, where it mediates the repressor function of Hairless as well as of the Enhancer of split bHLH proteins, the principle nuclear effectors of Notch signalling (e.g., [18,19]). In fact, these data provided a first molecular node for the long-standing genetic relationship between the EGFR and Notch signalling pathways in *Drosophila*. Genetic interactions between both pathways have been extensively documented, demonstrating that they sometimes are cooperative; however, mostly they are antagonistic (for a review, see [2–4]). This ambivalent relationship can be observed for example in the context of tumorigenesis, which both pathways profoundly affect. In pancreatic cancer, EGFR activity is correlated with an increased Notch signalling activity, providing an example for a cooperative interplay in this pathogenesis [20]. Accordingly, a downregulation of Notch activity during the imaginal development of *Drosophila* is accompanied by a reduced EGFR signalling activity and apoptosis [21]. Notch may also act as a tumour suppressor by inhibition of receptor tyrosine kinase-mediated tumour cell proliferation [22–24]. Such opposing effects can also be observed during the establishment of wing veins and bristles in *Drosophila*. In both cases, EGFR signalling promotes vein and bristle cell fate by counteracting the Notch pathway in a strictly defined spatio-temporal manner [25,26].

Here we show that Su(H) contains a MAPK phosphorylation site in the C-terminal part of the protein which is phosphorylated *in vitro* and *in vivo* in response to MAPK activity. This modification provokes an altered Notch signalling output, as a constitutively pseudo-phosphorylated form of Su(H) [$Su(H)^{MAPK\text{-}ac}$] reduces Notch signalling activity, whereas a phospho-deficient Su(H) [$Su(H)^{MAPK\text{-}ko}$] isoform enhances it. Our analyses therefore suggest a new molecular junction between the EGFR and Notch pathways at the level of the Notch mediator Su(H).

2. Materials and methods

2.1. Generation of Su(H) overexpression constructs and flies

In vitro mutagenesis of Su(H) cDNA was performed using the QuikChange XL mutagenesis kit (Stratagene, La Jolla CA, USA) to yield $Su(H)^{MAPK\text{-}ko}$ (T426A) and $Su(H)^{MAPK\text{-}ac}$ (T426E). Constructs were sequence verified and shuttled into respective vectors, pJG, pMT and pUAST. Transgenic UAS-fly lines were generated following standard protocols. One representative line each [$Su(H)^{MAPK\text{-}ac79}$ and $Su(H)^{MAPK\text{-}ko114}$] out of ten independent transgenic fly lines was used for further analyses based on similar expression activity as determined by Western blots using anti-Su(H) [45] and anti-beta tubulin antisera (DSHB) for loading control. To this end, respective lines were expressed in the developing eye using gmr-Gal4, and protein extracts were prepared from 20 fly heads each.

2.2. Overexpression and clonal analyses

Clones lacking MAPK function were generated as described earlier [17] using *y w* $flp^{1.22}$; P{neo FRT}82B $ras85D^{x7b}$/TM6B and w^{1118}; P{neo FRT}82B P{Ubi-GFP(S65T)nls}3R/TM6B (kindly provided by Z. Paroush) [17]. Overexpression clones were induced by the flip-out technique [46] with the following fly lines: *y w* $flp^{1.22}$; UAS-Su(H) or UAS Su(H) mutants, *y w* $flp^{1.22}$; UAS-rl^{SEM} and *y w* $flp^{1.22}$; UAS-rl^{SEM} UAS-Su(H) and *y w* Act > CD2 > Gal4, UAS-GFP-nls (kindly provided by K. Basler). Tissue-specific overexpression was achieved using the Gal4/UAS-system [33]. As driver lines, we used Bx-Gal4, en-Gal4, Eq1-Gal4 (kindly provided by H. Sun) and QE-Gal4. QE-Gal4 was generated by PCR-amplifying the *vestigial* quadrant-enhancer element [47] from genomic fly DNA (genotype cn^{1}; ry^{506}) and inserting it into pGATB (obtained from DGRC). As effector lines UAS-Su(H), UAS-$Su(H)^{MAPK\text{-}ac}$, UAS-$Su(H)^{MAPK\text{-}ko}$, UAS-rl^{SEM} (kindly provided by E. Martín-Blanco) [44], UAS-DER^{act} (kindly provided by M. Freeman) [43], UAS-HFL, UAS-N^{ICN} and UAS-ras^{V12} were used. For information on fly stocks, we refer to http://flybase.bio.indiana.edu. Flies were obtained from the Bloomington stock collection if not noted otherwise. Fly husbandry was according to standard protocols at 25 °C or 18 °C as noted.

2.3. Immunohistochemistry

In vivo expression of Notch target genes or controls was determined according to standard protocols by using mouse monoclonal antibodies directed against Cut, Wingless, beta-Galactosidase or GFP (developed by G. M. Rubin, S. M. Cohen and J. R. Sanes, respectively, and obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biology, Iowa City, IA 52242). Secondary antibodies coupled to DTAF, Cy3 or Cy5 (1:200) were obtained from Jackson Immuno-Research Laboratories (Dianova, Hamburg, Germany).

2.4. Analyses of Su(H) DNA and protein binding properties

DNA binding of Su(H) protein variants was tested by electromobility shift assays using the m8-S1 oligo that contains a single Su(H) binding site as described earlier [29,30]. Su(H) proteins were produced by in vitro translation using the TNT®Coupled Reticulocyte Lysate System (Promega). Titration was performed by adding increasing amounts of unlabelled oligo to the reaction and measuring the signal intensity in the autoradiogram by densitometry using the Aida Development program (2.01.53). Yeast two-hybrid and three-hybrid assays were performed as described before, using pEG-ICN I (covering RAM-ANK), pEG-HFL or pEG-MamN as bait, pESC-ANK as intermediate and pJG-Su(H) (wild-type or mutant) constructs as prey [39]. The expression of the proteins in the yeast cells was verified with mouse anti-HA antibodies or rabbit anti-LexA antibodies (1:1000) (both from Roche, Basel, Switzerland).

2.5. S2 cell culture and transfection assays

Schneider S2 cells (from DGRC) were maintained in Shields and Sang M3 medium (Sigma Aldrich, Munich, Germany) supplemented with fetal calf serum (Biochrom AG, Berlin, Germany) and transfected with pMT-Gal4, UAS-Su(H)-myc and pMT-ras^{V12} (kindly provided by I. Rebay) [31]. UAS-Su(H)-myc was generated by shuttling the Myc-tagged construct from pET-21A (kindly provided by M. Caudy) [48] into pUAST vector [33]. The expression of constructs was induced by adding 0.7 mM $CuSO_4$ and harvested 18-22 hours later. Alternatively, 150 nM bovine insulin [32] was added to the pMT-Gal4, UAS-Su(H)-myc transfected and induced cells 5 minutes before the harvest. Su(H) protein was immuno-precipitated from roughly 10^8 cells using anti-Myc antibodies (rabbit polyclonal, Santa Cruz Biotechnology, CA) and detected either with anti-Su(H) antibody (rat polyclonal) [45] or