WW domain–mediated regulation and activation of E3 ubiquitin ligase Suppressor of Deltex

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The Nedd4 family E3 ligases Itch and WWP1/2 play crucial roles in the regulation of cell cycle progression and apoptosis and are closely correlated with cancer development and metastasis. It has been recently shown that the ligase activities of Itch and WWP1/2 are tightly regulated, with the HECT domain sequestered intramolecularly by a linker region connecting WW2 and WW3. Here, we show that a similar autoinhibitory mechanism is utilized by the Drosophila ortholog of Itch and WWP1/2, Suppressor of Deltex (Su(dx)). We show that Su(dx) adopts an inactive steady state with the WW domain region interacting with the HECT domain. We demonstrate that both the linker and preceding WW2 are required for the efficient binding and regulation of Su(dx) HECT. Recruiting the multiple-PY motif–containing adaptor dNdfip via WW domains relieves the inhibitory state of Su(dx) and leads to substrate (e.g. Notch) ubiquitination. Our study demonstrates an evolutionarily conservative mechanism governing the regulation and activation of some Nedd4 family E3 ligases. Our results also suggest a dual regulatory mechanism for specific Notch down-regulation via dNdfip–Su(dx)–mediated Notch ubiquitination.

Ubiquitination controls the future of most proteins by regulating their location, degradation, and function. Ubiquitin ligases (E3s) catalyze the final step of the process by transferring ubiquitin (Ub)2 from ubiquitin-conjugating enzymes (E2s) to target proteins and, thus, determining the specificity of ubiquitination (1). The Nedd4 family members are the largest subgroup of HECT (homologous with E6-associated protein C terminus)-type E3 ligases and play diverse roles in regulating cell signaling (e.g. Notch, IGF-1, transforming growth factor β, and bone morphogenetic protein) and endocytosis and sorting of numerous transmembrane proteins (e.g. epithelial sodium channel and LAPTM5) and various aspects of the viral life cycle (2, 3). As Nedd4 family members are involved in the degradation of many tumor promoters or suppressors, the aberrant activity of Nedd4 E3s has been frequently identified in human cancers, immune disorders, and other diseases (2, 4–7).

All nine members of the mammalian Nedd4 family E3s (Itch, WWP1/2, Nedd4/4L, Smurf1/2, and NEDL1/2) share a common domain architecture composed of a C-terminal catalytic HECT domain involved in E2 binding and Ub transfer, an N-terminal C2 domain, and multiple WW domains responsible for subcellular localization and substrate recognition. To prevent nonphysiological ubiquitination of themselves and substrates, many Nedd4 family E3s are kept in an inactive state, although the underlying mechanisms are not clear yet. Known structural information of the Nedd4 family HECT domains in their isolated, E2-bound, and substrate-bound states revealed that the bilobal HECT domain (with the N- and C-lobes connected by a flexible hinge loop) can adopt distinct subdomain orientations during an ubiquitination cycle, whereas in full-length proteins, the N-terminal regulatory domains restrict the rotation of the two lobes to inhibit the ligase activity (8–10). The C2 domains in Smurf1/2 and Nedd4/4L interact with their HECT domains intra- or intermolecularly to suppress its E2-E3 transsthiolation and noncovalent Ub binding (11–14). Such C2–HECT autoinhibition could be relieved via C2 binding to Ca2+/phospholipids or adaptors (e.g. Cdh1), HECT binding to adaptors (e.g. Smad7), or posttranslational modifications on C2 and/or HECT (e.g. Src-mediated phosphorylation of Nedd4) (11, 13–16). However, recent kinetic studies have argued against the C2-mediated autoinhibition of Nedd4L and suggested a two-step proximal indexation model for polyubiquitin chain assembly (17, 18).

On the other hand, the autoinhibitory function of the WW domain region has been observed in several Nedd4 E3s, and, in some cases, the interaction was mediated by WW domain binding to the PY motif within the HECT domain (19–21). Recruitment of multiple PY-containing targets or adaptors, such as Nedd4 family–interacting protein 1/2 (Ndfip1/2), via WW domains could free the HECT domain (19, 20, 22, 23). Recently, two parallel studies provided another mechanism governing the WW–HECT interaction (24, 25). A linker region (hereafter referred to as L) connecting two WW domains of WWP2 (also WWP1, Itch, and Nedd4) can keep the HECT domain in an...
inactivating conformation by blocking the flexible hinge loop and the noncovalent Ub binding site (24). Such L-mediated inhibition could be relieved by the phosphorylation of L. Our recent work confirmed the requirement of L in the autoinhibition of Itch and WWP1/2 and suggested an essential role of WW2 (the adjacent WW domain ahead of L) in the regulation and activation of those E3s (25). Binding to the three-PY-bearing adaptor Ndfip1 or JNK1-mediated phosphorylation relieves the autoinhibition of Itch in a WW2-dependent manner (25, 26). Whether such L- or WWL-mediated regulation and activation mechanisms apply to other Nedd4 family ligases is currently unclear.

An evolutionarily conservative target of the Nedd4 family E3s from worms to humans is Notch (27–31), a cell surface receptor playing a key role in multiple fate decisions during development and the progression of many cancers (32–35). In mammals, Itch mediates the ubiquitination and degradation of Notch1 (30). Itch-deficient hematopoietic stem cells showed accelerated proliferation rates and sustained progenitor properties due to the accumulation of activated Notch1 (36). WW2 down-regulates Notch3 signaling in ovarian cancer by targeting Notch3 for lysosomal degradation (31). *Drosophila* Nedd4 (dNedd4) regulates the internalization of Notch and suppresses its ligand-independent activation by promoting the degradation of Notch and Deltex (27). Suppressor of Deltex (Su(dx)), an Itch-related protein in *Drosophila*, antagonizes Deltex to regulate the trafficking and degradation of Notch at a high temperature in its ligase activity-dependent manner (29, 37, 38). Itch, WWP2, dNedd4, and Su(dx) were all proposed to interact directly with the intracellular domain of Notch (referred to as NICD hereafter) proteins through their WW domains, although Notch1 does not contain a canonical PY motif (30, 31, 36, 39). Ndfip1/2 can increase the ligase activities of Itch (20, 22, 25), which leads to target ubiquitination and degradation (23). Interestingly, it has been reported that the *Drosophila* Ndfip (dNdfip) expression dramatically enhances the wing phenotypes of dNedd4 and Su(dx) by promoting their activity in Notch ubiquitination (40), implying that dNedd4 and Su(dx) may also be kept in an inactive steady state. However, whether dNedd4 and Su(dx) adopt a similar autoinhibitory mode as Itch and WWP1/2 and whether the dNdfip-mediated activation of dNedd4/Su(dx) is WW domain-dependent is unclear.

In this study, we demonstrate that similar to its mammalian orthologs, Su(dx) is kept in an autoinhibitory state with its catalytic HECT domain sequestered by the WW2L domain. Both WW2 and L are required for the efficient regulation of Su(dx) ligase activity. Human Ndfip1 can release the autoinhibitory state of Su(dx). dNdfip can also activate Su(dx) by binding to its WW domains, although only two of three PY motifs are required. We further find that dNdfip competes with Notch for Su(dx) WW domain binding, implying a dual regulatory role of dNdfip in Su(dx)-mediated Notch degradation.

**Results**

**L is essential for Su(dx) autoinhibition**

Su(dx) possesses the same domain organization as its mammalian orthologs Itch and WWP1/2, with a C2 domain and four WW domains ahead of the HECT domain (Fig. 1A). As expected, an *in vitro* Su(dx) autoubiquitination assay showed that the protein adopts an inactive conformation, and the C2 domain plays a minor role in its ligase regulation (Fig. 1, A–C), just as its mammalian orthologs do (24, 25). These data suggest that the WW domain region may be responsible for keeping Su(dx) at a low activity level.

Primary sequence analysis of these Nedd4 family proteins revealed that most key residues of WW2 and L involved in Itch HECT binding are highly conserved in Su(dx) (Fig. S1), implying that the L (or even WW2L)-mediated autoinhibition mode observed in Itch and WWP1/2 may also be utilized in Su(dx). We then confirmed this possibility in several ways. Combinatorial deletion of both C2 and L (1234HECT) caused a striking increase in Su(dx) autoubiquitination comparable with that of the isolated HECT domain (Fig. 1, A, C, and D), indicating that L indeed played a dominant role in the autoinhibition of Su(dx). However, although essential, L alone seemed to be insufficient for a complete HECT inhibition, as LHECT possessed a dramatically increased ligase activity compared with that of the C2 deletion (12L34HECT) mutant (Fig. 1, A and C). A GST pull-down assay showed that GST-tagged HECT could only pull down MBP-tagged WW12L34 and WW12L and not the isolated L (Fig. 1E). Supporting these data, an analytical gel filtration assay showed that the HECT domain and WW12L34 could form a stable 1:1 stoichiometric complex with a pronounced early elution peak compared with that of the isolated HECT domain or WW12L34 (Fig. 2A). In contrast, the mixture of HECT and L was eluted as two separated peaks (Fig. 2B). Moreover, the ligase activity of Su(dx) HECT could be robustly inhibited by WW12L34, but not L, in a dose-dependent manner (Fig. 2, C and D). Taken together, these data suggested that L is essential but not sufficient for Su(dx) autoinhibition, and the WW domain(s) are likely involved in its ligase regulation.

**WW2 and L act synergistically to lock Su(dx) in an inactive state**

To determine which WW domain(s) is responsible for the autoinhibition of Su(dx), we created a series of deletion mutants of Su(dx) on the template of the inactive 12L34HECT and tested their ligase activities by an autoubiquitination assay (Fig. 1, A and C). Our results suggested that the deletion of WW34 (12LHECT) had negligible impact on the enzymatic activities of Su(dx), whereas the deletion of WW12 (L34HECT) led to a dramatic increase in Su(dx) autoubiquitination (Fig. 1C), indicating that L and WW12, but not WW34, are responsible for HECT inhibition. This assumption was further confirmed by the observation that L34HECT has enzymatic activity comparable with that of LHTECT. Moreover, Su(dx) HECT could form a complex only with WW12L and not LW12L34, although the HECT–WW12L complex was less stable than HECT–WW12L34, due to the instability and easy degradation of WW12L (Fig. 2, E and F). Consequently, the autoubiquitination of the isolated HECT domain could be efficiently inhibited by WW12L but not LW12L34 (Fig. 2, G and H). We further dissected the role of WW1 and WW2 in Su(dx) autoinhibition. The W1 deletion (2L34HECT) mutant barely had enzymatic activity, whereas the W2 deletion (1L34HECT) mutant had...
ligase activity comparable with that of LHECT (Fig. 1D), indicating that WW2 but not WW1 contributed to Su(dx) autoinhibition. Although MBP-tagged WW12L could be robustly pulled down by GST-HECT, the isolated WW1, WW2, or L could not (Fig. 1E), implying that WW2 cooperated with L to interact with HECT with high affinity. Taken together, these observations revealed that WW2 and L act synergistically to maintain Su(dx) in a closed conformation.

**The structural model of 12LHECT**

To gain detailed structural information of how WW2L regulates the ligase activity of Su(dx), we attempted to grow crystals of inactive Su(dx) with different constructs, including the full-length protein, 12L34HECT, 12LHECT, and 2LHECT. However, our extensive trials failed. Considering the high sequence conservation between Itch and Su(dx) (Fig. S1), we constructed a structural model of Su(dx) 12LHECT using the SwissModel (41) server based on the known autoinhibited crystal structure of Itch (PDB code 5XMC; Fig. 3A). In this model, WW2 interacts with the N-lobe, and L forms an α-helix tucking into a groove formed between the N- and C-lobes of HECT (24, 25). Detailed structural analysis of the Su(dx) 12LHECT model indicated that intramolecular packing may be driven by extensive hydrophobic and polar–polar interactions (Fig. S2). For instance, the hydrophobic residues Tyr452, Leu453, and Tyr454 from the C terminus of L pack with a hydrophobic patch formed by Phe574, Leu578, and Ile827 from the N-lobe of HECT and Met831 from the hinge loop, whereas Met654 from the N-lobe inserts into a small pocket formed by Trp401 from WW2 and Phe434 from L. Salt bridges formed between Arg441 from L and Glu649 from the N-lobe of HECT, and hydrogen bonds formed between Arg531 from L and Glu832 from the hinge loop, which further strengthened the WW2L–HECT interaction.

Consistent with the above structural model, mutations of these key residues from either L (R441A, R451A, and Y454A), WW2 (W401A), or HECT (F644A, M654A, and M831A/Q832A) all led to disrupted or severely impaired autoinhibition of Su(dx) (Fig. 3, B and C). It was reported that the WW2L HECT binding–induced formation of the His830–Glu649 hydrogen pair is important for keeping Itch in its closed conformation.

Figure 1. Su(dx) E3 ligase activity is autoinhibited. A, schematic of Su(dx) domains with residue numbers indicated. B–D, autoubiquitination assay of the MBP-tagged full-length Su(dx) and various fragments. Reactions were quenched at 15 min for B and D and 15 or 30 min for C.

Statistics of enzymatic activities are shown below. Data are presented as the mean ± S.D. (error bars) of the triplicate experiments. E, GST pulldown assay of MBP-tagged WW12L34, WW12L, WW1, WW2, and L with GST-HECT. MBP-L is indicated with an asterisk.
WW-mediated autoinhibition and activation of Su(dx)

A

B

C

D

E

F

G

H
As His^{830} and Glu^{639} are completely conserved in Su(dx) (Fig. S1), we next tested whether such His/Glu hydrogen bonding may also contribute to the autoinhibited conformation of Su(dx). Indeed, a substitution of Glu^{724} (corresponding to Glu^{639} in Itch) to Ala resulted in a significant increase in Su(dx) autoubiquitination, and a triple mutation E724A/M831A/Q832A (referred to as EMQ/A hereafter) further increased the ligase activity (Fig. 3, B and C). Taken together, the above structural model and biochemical data demonstrated that the stabilization of the closed HECT conformation resulting from WW2L binding is the origin of the autoinhibited enzymatic activity of Su(dx) (Figs. 1–3).

**dNdfip binding to WW domains activates Su(dx)**

Su(dx) can mediate Notch ubiquitination and thus downregulate Notch signaling by directly interacting with the PY motif in NICD through its WW4 domain (Fig. 4A) (29, 37, 38). However, the ligase activity of Su(dx) toward Notch is normally

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**Figure 3. The simulation model of Su(dx) 12LHECT.** A, ribbon diagram of the structural model of Su(dx) 12LHECT generated by SwissModel using Itch (PDB code 5XMC) as the template. The coloring is the same as in Fig. 1A. The mutation residues are displayed as spheres. B and C, autoubiquitination assay of Su(dx) WT and various mutants. Ubiquitination efficiencies were normalized to those of Su(dx) WT and were plotted as the mean ± S.D. (error bars) of the triplicate experiments.

**Figure 2. WW2 cooperates with L to regulate Su(dx) activity by binding to HECT.** A, analytical gel filtration assay showing that MBP-tagged WW12L34 can form a stable complex with MBP-HECT. The elution profile of globular markers is indicated with a dashed gray line. B, the isolated L could not form a complex with HECT. C, WW12L34 can inhibit the autoubiquitination of Su(dx) HECT in a dose-dependent manner. D, Su(dx) HECT autoubiquitination assay with different concentrations of MBP-L E and F, analytical gel filtration assay showing that WW12L (E) but not LWW34 (F) can form a stable complex with HECT. G and H, Su(dx) HECT ubiquitination assay in the presence of WW12L (G) or LWW34 (H). For the ubiquitination assay, reactions were quenched at 5 or 10 min. Data are presented as the mean ± S.D. (error bars) of triplicate experiments; ns, not significant; ***, p < 0.001; ****, p < 0.0001 using two-way analysis of variance with Dunnett’s multiple-comparison test. Asterisks, MBP- or GST-tagged HECT.
**WW-mediated autoinhibition and activation of Su(dx)**

A

![Diagram](image)

B

dNdfp 22 SAPPFY<40>ADLMNAQLPP-E-IHGLPTYE<245>LPPRNPLL 120
hNdfp1 37 DAPPPYSSISAESAAYFDYKDESFGPKPPSYNVATLPSYDEAER 81

C

| Time(min) | Su(dx) | hNdfp1 |
|-----------|--------|--------|
| 0         | 0      | 0      |
| 30        | 0      | 0      |
| 60        | 0      | 0      |

Unmodified Su(dx) %

D

| Time(min) | dNdfp | Su(dx) |
|-----------|-------|--------|
| 0         | 0     | 0      |
| 30        | 0     | 0      |
| 60        | 0     | 0      |

Unmodified Su(dx) %

E

MBP-WW12L34
MBP-WW12L
MBP-LWW34
GST-dNdfp
GST-Notch PY

Input

GST Pull-down

F

Time (min)

0 10 20 30 40

Cal/sec

Kcal/mol of injection

N=1.9
Kd=5.6 ± 0.7 µM

Kd=2.2 ± 0.2 µM

G

MBP-12L34 HECT
MBP-12L4 HECT
MBP
Trx-Ndfp

Input

MBP Pull-down

H

MBP-12L34 HECT
MBP-EMQ/A
MBP
Trx-Ndfp

Input

MBP Pull-down

Ndfp/Su(dx) ratio

0.7 0.6 1.9 1.0 2.1 1.2
low and can be boosted under certain circumstances (e.g. high temperature) or in the presence of adaptor proteins, such as dNdfip (38, 40). Ndfip1 and -2, each containing three typical PY motifs (Fig. 4B), bind WW domains and activate mammalian Nedd4 family E3s (22). Importantly, all three PY–WW interactions are required for Ndfip1/2 to bind to multiple WW domains with high affinity and selectivity, thus releasing Nedd4 E3s from an autoinhibitory intramolecular interaction (20, 22, 25). In combination with our in vitro autoubiquitination assay, it is possible that dNdfip can activate Su(dx) from its autoinhibited inactive state by competing with HECT for WW binding.

To test the above hypothesis, we first tested whether Su(dx) could be activated by human Ndfip1 (hNdfip1), in consideration of the high sequence conservation of Su(dx) with Itch and WWP1/2 (Fig. S1) (37). As expected, hNdfip1 was able to significantly increase the autoubiquitination of Su(dx) (Fig. 4C), although less efficiently than that of Itch (25). Although the sequence identity between dNdfip and hNdfip1 is quite low, dNdfip also contains three PY motifs in its N-terminal cytoplasmic domain (Fig. 4B), and each of them was required for the efficient activation of dNedd4 (27). Similarly, a Su(dx) autoubiquitination assay showed that dNdfip was able to activate Su(dx) robustly in a PY-dependent manner. Substitution of the PY1 or PY2 motif of dNdfip to alanine (PY1/A and PY2/A, respectively) significantly impaired its ability to activate Su(dx), whereas the PY3/A mutant possessed an ability to activate Su(dx) comparable with that of the WT protein (Fig. 4D). Therefore, different from its mammalian orthologs in which all three PY motifs are essential for Itch activation, only the PY1 and PY2 motifs of dNdfip are required and sufficient for releasing the autoinhibition of Su(dx).

As there are four WW domains in Su(dx), we next wanted to verify which WW domains are involved in dNdfip binding. A GST pulldown assay showed that dNdfip could robustly pull down MBP-tagged WW12L34, WW12L, and LWW34 (Fig. 4E), indicating that the PY1 and PY2 motifs of dNdfip might cooperatively interact with either WW12 or WW34 of Su(dx). ITC-based measurement confirmed that each Su(dx) WW12L34 could recruit two dNdfips with a micromolar binding affinity (Fig. 4F). In addition to free WW domains, the MBP-tagged autoinhibited Su(dx) 12L34HECT could pull down up to 2 molar ratios of dNdfip, whereas the WW3 deletion mutant (12L4HECT) could only bind to dNdfip with a 1:1 molar ratio (Fig. 4G). Note that the autoinhibited Su(dx) showed a slightly weaker binding property toward dNdfip than the semi-open EMQ/A mutant (Fig. 4H), demonstrating that dNdfip indeed competed with HECT for WW domain binding. Taken together, the above data indicated that dNdfip is capable of binding to WW12 or WW34 domains in Su(dx) with little selectivity, and the binding to WW2 may relieve the intramolecular inhibition of Su(dx).

dNdfip-mediated activation of Su(dx) leads to Notch ubiquitination

As WW domains are responsible for targets (normally containing PY motifs) recognition, we wondered whether and how dNdfip would interfere with Su(dx)’s interaction and ubiquitination of Notch. In agreement with a previous report that Notch selectively interacts with the Su(dx) WW4 domain (with a $K_d \sim 45 \mu M$) (39), a Notch fragment containing the PY motif (hereafter referred to as Notch PY) could only interact with MBP-tagged WW12L34 and LWW34 but not WW12L (Fig. 5A). Both dNdfip and Notch can interact with Su(dx) LWW34, suggesting that dNdfip would compete with Notch for binding to Su(dx) WW domains. Moreover, Su(dx) WW domains bound much stronger to dNdfip than Notch ($K_d \sim 5 \mu M$ versus 45 \mu M; Fig. 4F). Our in vitro competition experiments further showed that a small amount of dNdfip can effectively compete with Notch PY for binding to Su(dx) WW34 or WW12L34, whereas only excess amounts of Notch PY (e.g., at a 12:1 molar ratio of Notch to dNdfip) can compete with dNdfip for binding to Su(dx) LWW34 (Fig. 5, A–C). However, Su(dx) WW12L34 could form a heterotrimer with both dNdfip and Notch when Notch was saturated (Fig. 5D). Note that Notch bound to Su(dx) equally well in either its open or closed form (Fig. 5E), and GST-dNdfip could only pull down Notch in the presence of Su(dx) WW12L34 (Fig. 5F). In this dNdfip–Su(dx)–Notch trimer, dNdfip was assumed to interact with WW12 of Su(dx), and Notch with WW4. Presumably, dNdfip-bound Su(dx) regained its ligase activity, which subsequently leads to the ubiquitination of the adjacent Notch. Consistent with our hypothesis, a substrate ubiquitination assay showed that Notch NICD could be more efficiently ubiquitinated by Su(dx) when dNdfip was present (Fig. 5G). As a positive control, NICD could also be ubiquitinated by Su(dx) mutants (R451A and EMQ/A) that have high enzymatic activities in the absence of dNdfip (Figs. 3 and 5G). Furthermore, the WW3 deletion mutant 12L4HECT, which could recruit dNdfip only through its WW12 but not WW34, showed a much stronger ubiquitinating efficiency toward NICD (Figs. 4G and 5H), possibly because of the disrupted competition between NICD and dNdfip for Su(dx). In summary, these results demonstrated that dNdfip acts as an adaptor in Su(dx)-mediated Notch ubiquitination by relieving Su(dx) from its autoinhibitory inactive state.

Discussion

Autoinhibition is a general character of Nedd4 family E3 ligases, although the underlying molecular mechanisms differ (8). Rsp5, the only Nedd4 family member in yeast, undergoes a ubiquitination-dependent oligomerization of the HECT domain to inactivate the ligase (42). In mammals, some Nedd4 members (e.g. Smurf1/2 and Nedd4/4L) keep the enzymes in their low-activity state through binding of the C2 domain to the
HECT domain either intra- or intermolecularly. Molecules such as Ca\(^{2+}\)/phospholipids or posttranslational modifications that disrupt the C2–HECT interaction can activate those E3s, whereas other molecules (e.g., Itch, WWP1/2, and Nedd4) maintain autoinhibition by their WW domain region (including linkers connecting WW domains) interacting with the HECT domain (19, 20, 24, 25). This type of autoinhibition can be disrupted by multiple PY-containing adaptors (e.g., Ndfip proteins), clustered single-PY motif–containing substrates that compete with HECT for WW domain binding (25, 43, 44), or phosphorylation of the linkers (24). Here, we demonstrated that Su(dx), the Drosophila ortholog of Itch and WWP1/2, adopts a WW domain–mediated autoinhibitory conformation in which its WW2L locks the HECT domain in a closed state. dNdfip binding to Su(dx) WW2L frees the HECT domain to an active state and consequently leads to target (e.g., Notch) ubiquitination (Fig. 6).

**Figure 5. dNdfip-mediated activation of Su(dx) led to Notch ubiquitination.** A–D, an MBP bead pulldown experiment was used to assay the formation of Su(dx)–dNdfip or Su(dx)–Notch PY complexes. A and B, a small amount of dNdfip can compete with Notch PY for binding to Su(dx) WW34 (A) or WW12L34 (B). In these experiments, the amounts of MBP-Su(dx) proteins and GST-Notch PY in each assay were fixed at 8 and 40 μM, respectively, and the concentrations of Trx-dNdfip were gradually increased. C, only an excess amount of Notch PY could compete with dNdfip in binding to Su(dx) WW12L34. D, Su(dx) WW12L34 can form a heterotrimer with Notch PY and dNdfip when Notch is saturated. In these experiments, the total amounts of MBP-Su(dx) proteins and Trx-dNdfip were fixed (8 μM), and the concentrations of GST-Notch PY were increased to as high as 160 μM. E, MBP pulldown assay of MBP-tagged Su(dx) 12L34HECT or WW12L34 and GST-Notch PY. F, GST pulldown assay of GST-dNdfip and MBP-Su(dx) WW12L34 with or without a saturating amount of MBP-Notch PY. Notch PY pulled down by GST-dNdfip is indicated with an asterisk. The molar ratio of dNdfip, Su(dx), and Notch was 1:1:0 or 1:1:20. G and H, FLAG-Notch NICD ubiquitination by Su(dx) 12L34HECT WT or mutants (R451A, EMQ/A, and 12L4HECT) in the presence or absence of dNdfip. Ubiquitination efficiencies were normalized to those at the 0 min time point and were plotted as the mean ± S.D. of triplicate experiments on the right.
The α-helical linker connecting WW domains serves as a vital regulator of enzymatic activities of several Nedd4 members by restraining the HECT hinge loop and, thus, blocking subdomain rotation (24, 25). However, our results on Su(dx) suggest that, although essential, L alone seems to be not sufficient to lock these E3s in a completely inactive state due to its low binding affinity toward the HECT domain. The combination with an adjacent weak HECT-binding WW domain could dramatically strengthen the role of the linker in the binding and subsequent regulation of the HECT domain. The cooperative manner of WW2 and L may also be utilized in Itch and WWP2. In Itch, L could robustly pull down HECT only when combined with WW12, and mutations from the HECT-packing interface of WW2 dramatically increased the ligase activity of Itch (25). In WWP2, although not mentioned, the WW2LHECT construct seemed to adopt a more stable closed state than that of LHECT (24). Whether the WW domain preceding the identified L is involved in ligase regulation in WWP1 and Nedd4 needs further investigation. More efforts are needed to identify other examples of WW-L–mediated autoinhibition of Nedd4 family members. Moreover, in addition to the WW domain ahead of L, it is not known whether the following WW domain can cooperate with L for ligase regulation, which may provide new hot sites of HECT for ligase regulation.

Compared with L-mediated regulation of Nedd4 family E3s, the combined WW and L regulatory mode provides more diverse activation mechanisms. As shown in Itch/Su(dx), the HECT-packing interface on the WW2 domain is partially overlapped with the canonical PY motif–binding site. Presumably, PY motif binding to the regulatory WW domain displaces WW from HECT through steric hindrance; then the adjacent L dissociates from the HECT due to instability. As a result, in addition to postmodification on L, at least some Nedd4 family E3s can be activated by adaptors or targets with multiple PY motifs. Su(dx) impinges on Notch activity by interacting with the two PY–bearing scaffold Pyd via its WW12 domains (45). Su(dx) targets the YAP pathway protein Pez for degradation and modulates fly midgut homeostasis via its WW domains and Pez’s PY and PPPY motifs (46). In addition to PY-mediated activation, postmodification of Nedd4 E3s might generate WW-binding fragments to compete with the HECT domain intramolecularly. For example, JNK1-mediated phosphorylation of the Itch PRR region might generate WW-binding p(S/T) motifs and consequently displace WW2 from the HECT domain of Itch (25, 26).

In our dNdffip–Su(dx)–Notch axis, we found that dNdffip not only activates Su(dx) but also can compete with Notch for Su(dx) binding. This finding may have important significance to ensuring selective target ubiquitination. Although membrane tethering of some single-PY–bearing targets can activate certain Nedd4 E3s by achieving a high local density of PY motifs (43), this mechanism may be not applicable for the Notch–Su(dx) case, as Notch PY selectively binds to Su(dx) WW4 (39), and Su(dx) showed low activity toward membrane-tethered full-length Notch at a low temperature (38). On the other hand, due to the relative low selectivity and affinity of the WW–PY interaction, multiple-PY motif–containing proteins (e.g. Ndfip proteins) would bind much stronger to tandem WW domains than targets with a single PY motif. Thus, even when E3 (Su(dx)), adaptor (dNdffip), and target (Notch) meet, Notch signaling within the physiological (low) level will not be downregulated. However, under certain conditions (e.g. high temperature, which might induce the aggregation/accumulation of Notch), ectopically accumulated Notch will effectively compete with dNdffip for Su(dx) WW4 binding, which can then be ubiquitinated and degraded. In this scenario, the ubiquitination specificity would have two levels of regulation.

Materials and methods

Protein expression and purification

Various Drosophila Su(dx), Ndfip, Notch, and human Ndfip1 fragments (Fig. 1 and Table S1) were individually cloned into pGEX-6P-1, pMalc2X, or a modified version of pET32a vector (47), with the resulting protein containing a GST, MBP, or Trx tag followed with a His6 tag in its N terminus. The fragment of Notch NICD (amino acids 1760–2350) was cloned into pcMVtag2B vector, with the resulting protein containing a
WW-mediated autoinhibition and activation of Su(dx)

FLAG tag in its N terminus. All of the mutations were created through a standard PCR-based mutagenesis method and confirmed by DNA sequencing. Recombinant proteins were expressed in *Escherichia coli* BL21 (DE3) host cells at 16 °C and were purified by using nickel-nitritoltriacetic acid–agarose affinity chromatography followed by size-exclusion chromatography.

**In vitro ubiquitination assay**

For the autoubiquitination assay, 800 nm Su(dx) proteins were incubated in 50 μl of buffer A (containing 60 nm E1 (UBE1), 400 nm E2 (UBCH5A), 20 μM HA-Ub, 50 mm Tris (pH 7.5), 5 mM MgCl₂, 1 mM DTT, and 5 mM ATP) at 37 °C. For the ubiquitination assay, E1, E2, and E3 were all freshly purified for each experiment. Thus, the enzyme concentrations were based on the protein concentrations. For hNdfip or dNdfip-mediated activation of Su(dx), Su(dx) was preincubated with dNdfip or hNdfip with a 1:2 molar ratio or an indicated amount. The reactions were initiated by adding ATP and carried out at 37 °C.

For the substrate ubiquitination assay, FLAG-Notch NICD was expressed in HEK293T cells, purified by anti-FLAG M2 affinity gel (Sigma), and then eluted with FLAG peptide. Purified FLAG-Notch was incubated with 800 nm Su(dx) WT or mutants, with or without 1.6 μM dNdfip in 50 μl of buffer A.

Reactions were quenched by mixing the reaction mixture with SDS loading dye at the indicated time points, and samples were then resolved by SDS-PAGE, stained with Coomassie brilliant blue (CBB), or used for immunoblotting. The unmodified Su(dx) or substrate bands at distinct time points shown in the figures were quantified and normalized to the zero time points. All assays were repeated three times showing similar results.

**Immunoblotting**

The reaction mixtures were subjected to SDS-PAGE and then transferred to 0.45–μm polyvinylidene difluoride membranes (Millipore). Membranes were blocked using 3% BSA in TBST (20 mM Tris-HCl (pH 7.4), 137 mM NaCl and 0.1% Tween 20) buffer at room temperature for 1 h, followed by incubation with HA (ABclonal) antibody at a 1:5,000 dilution or anti-FLAG (Santa Cruz Biotechnology, Inc.) antibody at a 1:5,000 dilution or FLAG antibody at a 1:3,000 dilution or HA (ABclonal) antibody at a 1:3,000 dilution or FLAG peptide. Puriﬁed FLAG-Notch was incubated with 800 nm Su(dx) WT or mutants, with or without 1.6 μM dNdfip in 50 μl of buffer A.

**ITC measurements**

ITC measurements were performed on an ITC200 Micro calorimeter (MicroCal) at 25 °C. All of the protein samples were dissolved in buffer B. The titrations were carried out by injecting 40-μl aliquots of dNdfip (0.5–0.7 mM) into Su(dx) WW12L34 or WW34 (0.03–0.05 mM) at time intervals of 2 min to ensure that the titration peak returned to the baseline. The titration data were analyzed using the program Origin version 7.0 and ﬁtted by the one-site binding model.

**Protein structure modeling**

The structural model of Su(dx) WW12LHECT (amino acids 363–470 + 556–949) was generated by online server Swiss-Model (41) using the crystal structure of mouse Itch (PDB code 5XMC) as a template, in combination with the sequence alignment of Su(dx) with Itch performed by Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/)³ (48). The final model had 94.8% of the residues in the favored region of the Ramachandran plot. The three-dimensional structure of Su(dx) WW12LHECT was analyzed using PyMOL.

**Analytical gel filtration assay**

Analytical gel filtration experiments were carried out on an AKTA FPLC system (GE Healthcare). Proteins (20 μM, 100 μl) were loaded on a Superdex™ 200 Increase 10/300 GL column (GE Healthcare) equilibrated with buffer B.

**Competition assay**

MBP-tagged Su(dx) WW12L34 or LWW34 was first loaded onto MBP beads and then incubated with the indicated amount of potential binding partners in 500 μl of buffer B for 1 h at 4 °C. After washing three times, proteins captured by affinity beads were eluted by boiling, resolved by 10% SDS-PAGE, and detected by CBB.

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