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Postsynaptic requirement for Abl kinases in assembly of the neuromuscular junction

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Agrin signals through the muscle-specific receptor tyrosine kinase (MuSK) to cluster acetylcholine receptors (AChRs) on the postsynaptic membrane of the neuromuscular junction (NMJ). This stands as the prevailing model of synapse induction by a presynaptic factor, yet the agrin-dependent MuSK signaling cascade is largely undefined. Abl1 (previously known as Abl) and the Abl1-related gene product Abl2 (previously known as Arg) define a family of tyrosine kinases that regulate actin structure and presynaptic axon guidance. Here we show that the Abl kinases are critical mediators of postsynaptic assembly downstream of agrin and MuSK. In mouse muscle, Abl kinases were localized to the postsynaptic membrane of the developing NMJ. In cultured myotubes, Abl kinase activity was required for agrin-induced AChR clustering and enhancement of MuSK tyrosine phosphorylation. Moreover, MuSK and Abl kinases effected reciprocal tyrosine phosphorylation and formed a complex after agrin engagement. Our findings suggest that Abl kinases provide the developing synapse with the kinase activity required for signal amplification and the intrinsic cytoskeletal regulatory capacity required for assembly and remodeling.

The neural synapse is a region of intense structural specialization at sites of target innervation. The presynaptic terminal holds an exocytic machinery dedicated to the packaging and release of neurotransmitter. The postsynaptic membrane contains a neurotransmitter receptor complex in direct apposition to the presynaptic specialization. This precise arrangement attests to the fundamental role of the synapse in neurochemical transmission and its origin in a complex developmental program[1].

The NMJ is a prototypical synapse that is remarkable for its abundance and accessibility[1]. It is also a locus of inherited and acquired diseases, as seen in the congenital myasthenic syndromes and myasthenia gravis, respectively[2]. As a model of synapse formation, the NMJ offers the distinct advantage that a general mechanism of postsynaptic assembly is known. Notably, an organizing factor that is derived from the presynaptic nerve has been identified. This factor is agrin, a glycoprotein of ~400 kDa that is secreted by motor neurons and concentrated in the synaptic cleft. Agrin engages the postsynaptic MuSK in tandem with a proposed muscle-associated specificity component; this complex transduces a signal that leads to AChR clustering at densities approaching 10,000 molecules/μm^2 (ref. 1). Gene ablation studies have shown that both agrin and MuSK are required for functional synapse formation[1], although some patterning of muscle AChR gene expression has been observed in the absence of the former[3,4]. MuSK has an intrinsic tyrosine kinase activity that is required for AChR clustering, and MuSK is phosphorylated on multiple tyrosine residues after agrin engagement. Specifically, tyrosine phosphorylation of residues in the kinase activation loop and the juxtamembrane region of MuSK is required for agrin-induced AChR clustering[5–7]. Data from the crystal structure of the MuSK cytoplasmic domain indicate that such phosphorylation may relieve an autoinhibitory conformation in which the activation loop obstructs the active site[8].

Despite the requirement for its activity, MuSK is not thought to be a direct effector of receptor clustering. This is because MuSK tyrosine phosphorylation can be experimentally separated from downstream events such as tyrosine phosphorylation of AChR subunits[9]. Instead, MuSK is thought to initiate a tyrosine kinase signaling cascade. Candidate components of this cascade include the Src family kinases. Src and Fyn associate with MuSK and the AChR complex and are activated after agrin engagement[10]. Gene ablation and pharmacologic inhibition have, however, demonstrated that Src family kinase activity is not required for agrin-induced AChR clustering[11]. Thus a required nonreceptor tyrosine kinase (NRTK) activity downstream of agrin and MuSK has been long posited, but no such kinase has been identified to date.

In addition to an undefined tyrosine kinase signaling cascade, agrin-induced AChR clustering seems to require cytoskeletal reorganization. Agrin stimulation activates the small guanine triphosphatases (GTPases) Rac and Cdc42 (ref. 12) and the serine/threonine kinase Pak[13]. These three enzymes make up a cytoskeletal regulatory machinery implicated in membrane ruffling and formation of lamellopodia and filopodia in many cell types[14]. Agrin stimulation also results in actin polymerization and translocation of the actin-regulatory protein cortactin to sites of postsynaptic assembly[15]. Moreover, inhibitors of actin polymerization such as latrunculin A block agrin-induced AChR clustering in cultured myotubes[15].

Abl1 (also known as Abl) and Abl2 (also known as Arg) define a family of NRTKs characterized by unique carboxy-terminal actin-binding domains[16–18]. Abl kinases transduce signals downstream of growth factor stimulation[19] and integrin engagement[20] and shape the

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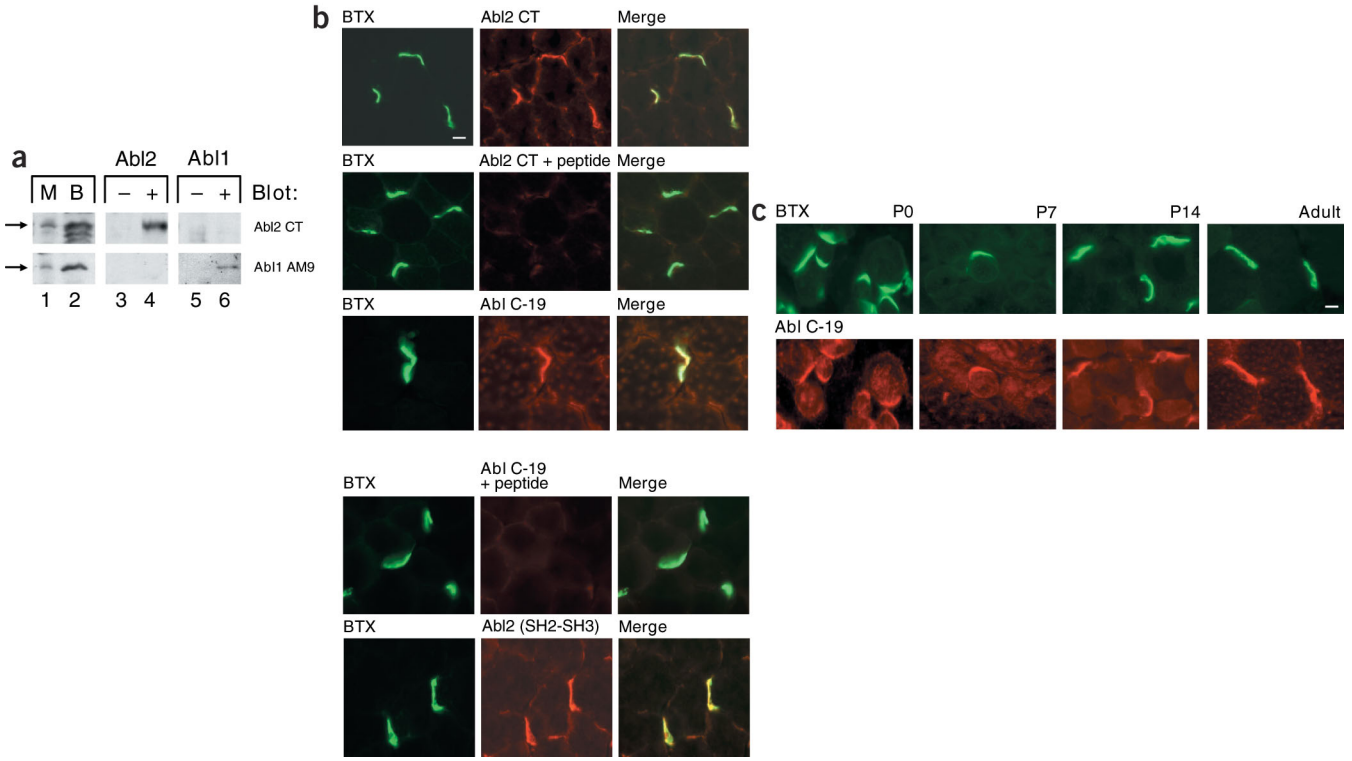

Figure 1 Abl kinases are expressed in mouse muscle and are localized to the developing NMJ. (**a**) Immunoblotting with antibodies specific for Abl2 (Abl2 CT) or Abl1 (Abl1 AM9) of lysates from mouse muscle (lane 1), brain (lane 2), *Abl1*$^{-/-}$*Abl2*$^{-/-}$ fibroblasts reconstituted with vector control (lane 3) or Abl2 (lane 4) and 293T cells transfected with vector control (lane 5) or Abl1 (lane 6). (**b**) Cross-sections of adult mouse tibialis anterior muscle costained with α-bungarotoxin (BTX, green), which binds postsynaptic AChRs, and antibodies to Abl kinases (red), alone or in the presence of immunizing peptide, as indicated. Scale bar, 10 μm. (**c**) Similar sections prepared from mice at postnatal days 0 (P0), 7 (P7), 14 (P14) and ~90 (Adult) stained with BTX and Abl C-19.

cytoskeleton through phosphorylation of regulatory proteins[16,21] and direct catalysis of actin bundling[17,18]. Although perhaps best known as oncoproteins underlying a subset of human leukemias[22], Abl1 and Abl2 are crucial for normal development[16,21,23–27]. In mice, *Abl1*$^{-/-}$ *Abl2*$^{-/-}$ (previously *Abl*$^{-/-}$ *Arg*$^{-/-}$) embryos die before 11 d postcoitum and suffer collapse of the neural tube secondary to disruption of neuroepithelial actin[24]. In *Drosophila melanogaster*, genetic ablation of the sole Abl family member (*D-Abl*) yields a well-characterized failure of axon guidance[16,21,25], but also early lethality[26] and epithelial defects, particularly in cell polarity[27] and formation of cell–cell junctions[28,29].

We hypothesized a role for Abl kinases in synapse formation, as synapses are, in essence, highly specialized cell-cell junctions[30] (not withstanding their capacity for neural transmission). We tested this hypothesis at the murine NMJ and here report the requirement for Abl kinases in the formation of this prototypical synapse. Abl kinases were expressed in mouse muscle and were localized to the postsynaptic membrane of the developing NMJ. In cultured myotubes, Abl kinase activity was increased by agrin stimulation and was required for agrin-induced AChR clustering and MuSK tyrosine phosphorylation. Furthermore, MuSK and Abl kinases effected reciprocal tyrosine phosphorylation and formed a complex upon agrin engagement. Our findings identify the Abl kinases as critical components of the tyrosine kinase signaling cascade downstream of agrin and MuSK and provide evidence of a postsynaptic function for this NRTK family in neural development.

RESULTS

Abl kinases localize to the developing NMJ

We first examined Abl family kinase expression in mouse muscle. *Abl2* transcripts are enriched in muscle[31], whereas expression of *Abl1* is

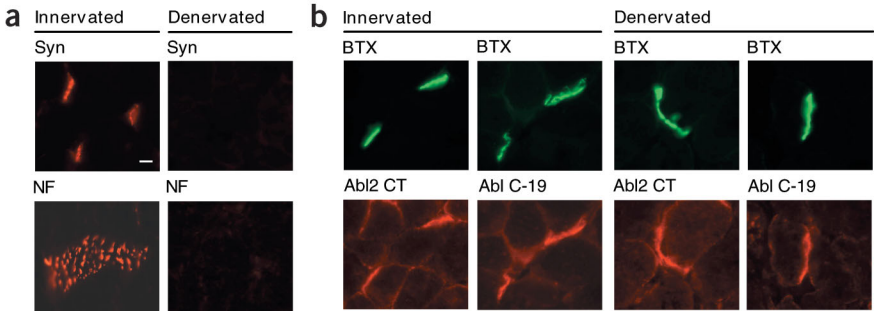

Figure 2 Abl kinases concentrate postsynaptically at the NMJ. (**a**) Surgically denervated mouse gastrocnemius muscle, 5 d post-operation (right column), or contralateral, innervated control (left column) stained independently for the presynaptic marker synaptophysin (Syn) and the axonal marker neurofilament (NF) or (**b**) co-stained with BTX and Abl2 CT or Abl C-19. Scale bar, 10 μm.

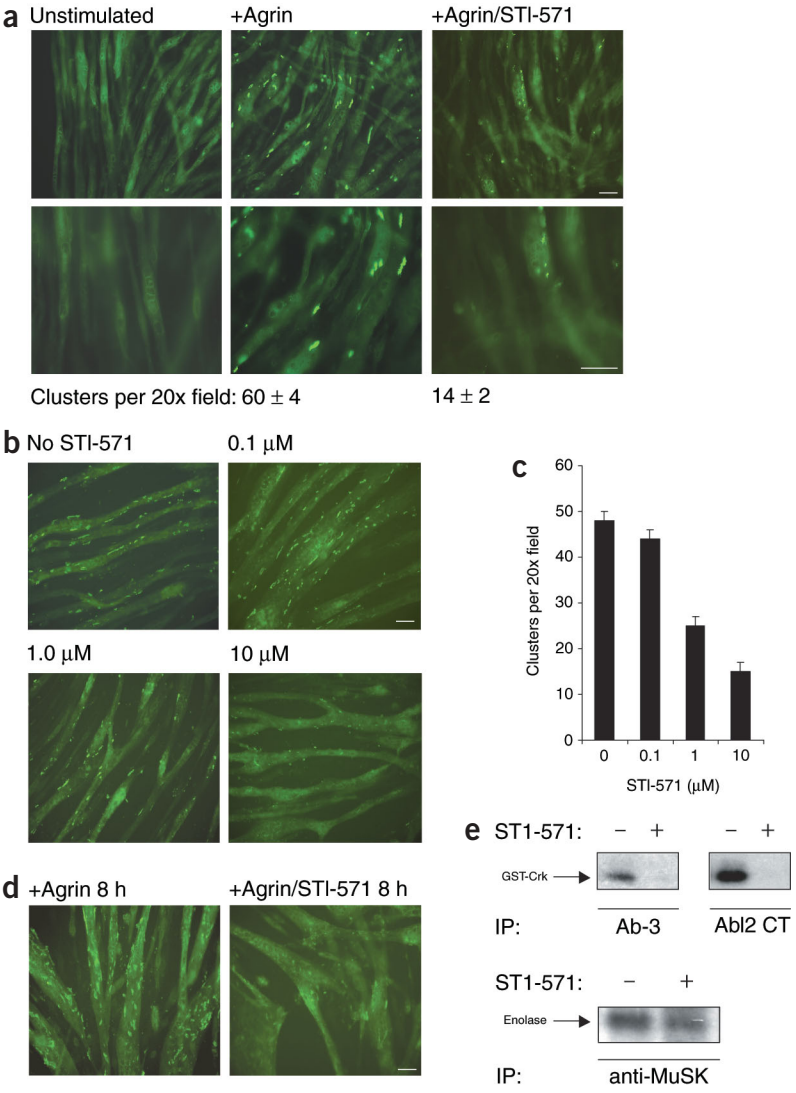

Figure 3 A specific pharmacologic inhibitor of Abl kinase activity blocks agrin-induced AChR clustering in cultured myotubes. (**a**) BTX staining (green) of C2C12 myotubes stimulated with 1 nM agrin in the presence of 10 μM STI-571 or vehicle control for 20 h. Additional magnification is provided in bottom row to emphasize differences in cluster size. Mean number of AChR clusters per field and s.e.m. are indicated. Differences were significant by Student's *t*-test ($P < 0.0001$). Quantitation of agrin-induced AChR clustering comprised counting all detectable clusters regardless of size in each of 25 randomly selected fields per sample at 20× magnification. Scale bar, 50 μm. (**b**,**c**) BTX staining of C2C12 myotubes stimulated with 1 nM agrin in the presence of varying concentrations of STI-571 for 20 h. Graph in **c** shows mean number of AChR clusters per field and s.e.m for each concentration. Differences were significant at 1 and 10 μM STI-571 by Student's *t*-test ($P < 0.0001$). (**d**) BTX staining of C2C12 myotubes stimulated as in **a**, except for reduction of incubation time to 8 h. (**e**) [^{32}P]γ-ATP incorporation into Abl substrate GST-Crk or *in vitro* MuSK substrate enolase after *in vitro* immune complex kinase assay with endogenous Abl1 (Ab-3), Abl2 (Abl2 CT) or MuSK (anti-MuSK) from C2C12 myotube lysates. Each pair represents a single lysate, split evenly and assayed identically in the presence of 10 μM STI-571 or vehicle control in the reaction cocktail. Note persistence of MuSK activity at inhibitor concentrations sufficient to obliterate Abl kinase activity. Similar results were obtained in six (**a**), three (**e**) or two (**b**–**d**) independent experiments.

ubiquitous[16]. Western blot analysis with antibodies to a specific C-terminal peptide of Abl2 (Abl2 CT) detected a discrete band of ~145 kDa in mouse muscle. This band migrated with both the largest Abl2 isoform in mouse brain and Abl2 that was overexpressed in cells (**Fig. 1a**). Similarly, antibodies specific for the first exon of Abl1 (Abl1AM9; ref. 32) detected a discrete band of ~145 kDa in mouse muscle. This band migrated with both the single Abl1 isoform in mouse brain and Abl1 that was overexpressed in cells (**Fig. 1a**). Thus, both Abl1 and Abl2 are expressed in mouse muscle.

We next determined the specific localization of the Abl kinases in mouse muscle. Abl2 CT antibodies and antibodies to a C-terminal peptide common to Abl2 and Abl1 (Abl C-19) stained the NMJ and could be outcompeted by excess antigen (**Fig. 1b**). We obtained similar results with antibodies to a portion of the Abl2 Src homology 2 and 3 domains, Abl2(SH2-SH3), that had previously been demonstrated to be Abl2 specific[24] (**Fig. 1b**). Thus, three independently generated, differently targeted antibodies to the Abl kinases stained the NMJ. Moreover, during development, Abl staining increased in intensity and complexity in concert with that of postsynaptic AChRs (**Fig. 1c**), which is a temporal pattern common to known regulators of synapse formation such as MuSK[33] and rapsyn[34].

Although Abl kinases have been implicated in presynaptic control of axon guidance[21,25], many facets of neuromuscular synapse formation suggest a postsynaptic function for this NRTK family. Specifically, agrin-induced AChR clustering requires activation of NRTKs that have not been identified[9], phosphorylation of multiple tyrosines in the MuSK receptor tail[5–7] and regulation of actin[15]. To determine whether Abl kinases have a postsynaptic localization that is consistent with such a function, we denervated mouse gastrocnemius muscle by sciatic nerve resection and compared Abl kinase localization in innervated and denervated tissue. Five days after nerve resection, presynaptic terminals had degenerated in stereotypic fashion, whereas postsynaptic AChR clusters remained (**Fig. 2a**,**b**). Abl C-19 and Abl2 CT staining persisted after denervation and colocalized with postsynaptic AChR clusters (**Fig. 2b**). Thus, Abl kinases at the NMJ concentrate postsynaptically, consistent with a role in agrin-dependent MuSK signaling.

Abl kinases mediate agrin-induced AChR clustering

To examine the role of Abl kinases in postsynaptic assembly at the NMJ downstream of agrin and MuSK, we used the C2C12 murine myoblast cell line. C2C12 myoblasts differentiate to myotubes under low-serum conditions and cluster AChRs efficiently when challenged with recombinant agrin[6,7], thus allowing the study of agrin-dependent MuSK signaling in the absence of additional presynaptic input. We treated C2C12 myotubes with recombinant agrin in the presence or absence of STI-571 (Gleevec), a specific inhibitor of Abl family kinase activity that is currently used to treat Bcr-Abl-positive leukemia[35,36]. We observed abundant clustering of

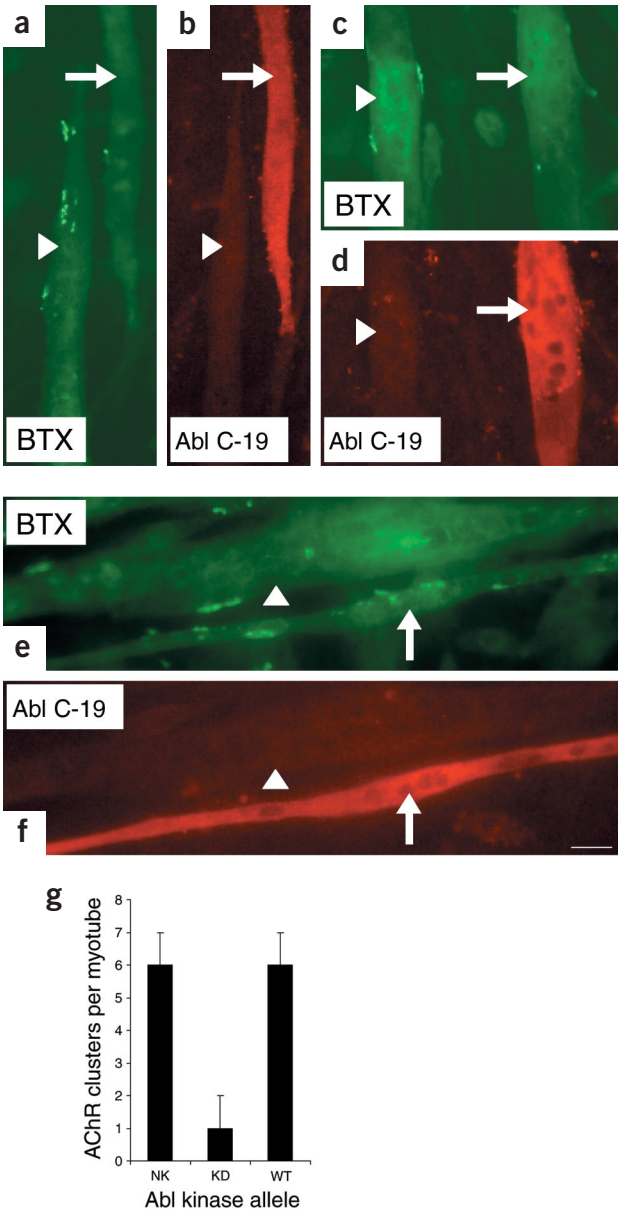

Figure 4 A kinase-defective *Abl1* allele blocks agrin-induced AChR clustering in C2C12 myotubes. (**a**–**f**) Staining with BTX (green) and Abl C-19 (red) of C2C12 myotubes overexpressing either kinase-defective (K290R; KD; ref. 19) in **a**–**d** or wild-type (WT) Abl1 in **e** and **f**, after stimulation with 1 nM agrin for 20 h. Arrows indicate transfected myotubes; arrowheads indicate untransfected controls. Scale bar, 20 μm. (**g**) Quantitation of agrin-induced AChR clustering in myotubes overexpressing KD ($n = 19$) or WT Abl1 ($n = 13$) or no exogenous kinase (NK; $n = 46$). Graph shows mean number of AChR clusters per myotube and s.e.m. The decrease observed in myotubes expressing KD Abl1 was significant (Student's *t*-test; $P < 0.001$).

AChRs in the presence of agrin (60 ± 4 clusters per 20× field; $n = 25$ fields), but this clustering was drastically reduced by treatment with STI-571 (14 ± 2 clusters per 20× field; $n = 25$ fields; **Fig. 3a**). Furthermore, clusters that did form in the presence of the Abl kinase inhibitor were uniformly undersized (**Fig. 3a**). Inhibition of agrin-induced AChR clustering by STI-571 was dose dependent (**Fig. 3b**,**c**), with an IC_{50} of approximately 1 μM. This is consistent with reported concentrations for inhibition of Abl kinase activity in cell culture[35,36]. In addition, STI-571-mediated inhibition was equally severe in a shorter incubation with agrin (**Fig. 3d**), suggesting an early block in the clustering process. We conclude that Abl kinases are required for membrane-proximal signal transduction downstream of agrin and MuSK. STI-571 had no effect on myotube survival or fusion, as determined by quantifying the number of myotubes per field and the number of nuclei per myotube, respectively (data not shown). In addition, STI-571 did not substantially inhibit MuSK activity in kinase assays at concentrations up to 400 times greater than the *in vitro* IC_{50} for Abl1[35] (**Fig. 3e**).

To confirm the requirement for Abl kinase activity in agrin-dependent MuSK signaling, we expressed a dominant-interfering, kinase-defective Abl1, as well as a wild-type Abl1 control, in C2C12 myotubes and examined the effect on agrin-induced AChR clustering. Given that Abl1 and Abl2 have greater than 90% identity in the SH2, SH3 and kinase domains[16], kinase-defective Abl1 should sequester binding partners and substrates that are common to both family members. We found that myotubes expressing kinase-defective Abl1 failed to cluster AChRs upon agrin challenge (**Fig. 4a–d**,**g**). In contrast, clustering occurred in all myotubes expressing wild-type Abl1 (**Fig. 4e–g**). In both cases, efficient clustering was observed in adjacent, untransfected myotubes. Thus dominant interference, like pharmacologic inhibition, reveals a requirement for Abl kinase activity in agrin-induced AChR clustering, probably in the context of membrane-proximal signal transduction.

An Abl kinase–MuSK signaling complex

Abl1 and Abl2 interact with multiple receptor tyrosine kinases (RTKs), including growth factor receptors[19], Eph receptor B2 (ref. 37) and TrkA[38,39]. Specifically, Abl kinases bind phosphotyrosine residues in the receptor tails of EphB2 and TrkA through their SH2 domains[37–39], and this binding facilitates tyrosine phosphorylation of Abl1 and Abl2 by EphB2 and vice versa[37]. Because Abl family kinase activity is required for agrin-induced AChR clustering (**Figs. 3** and **4**), we reasoned that Abl kinases and MuSK might interact similarly, particularly given the presence of multiple tyrosine phosphorylation sites in the intracellular domain of the latter[5,6]. We examined the functional relationship of Abl kinases and MuSK in the context of both transient coexpression in human embryonic kidney (HEK) cells and agrin stimulation of C2C12 myotubes. In HEK cells, expression of wild-type MuSK greatly increased the phosphorylation of Abl1 on tyrosine 245, whereas kinase-defective MuSK did not (**Fig. 5a**). As phosphorylation of Abl1 on tyrosine 245 is indicative of enhanced kinase activity[40,41], this indicates that MuSK may activate Abl1 in cells. Consistent with this, agrin stimulation of C2C12 myotubes increased endogenous Abl family kinase activity, as demonstrated by an increase in tyrosine phosphorylation of Crk, an endogenous Abl kinase-specific protein substrate[42,43] (**Fig. 5b**). Moreover, the time scale of Abl family kinase activation mirrored that previously reported for MuSK[9]. Expression of wild-type and constitutively active Abl1 in HEK cells greatly increased tyrosine phosphorylation of MuSK, whereas kinase-defective Abl1 did not (**Fig. 5c**). This indicates that activated Abl1 may, in turn, activate MuSK and/or enhance downstream signaling through creation of additional docking sites for phosphotyrosine-binding proteins. In this regard, agrin-induced tyrosine phosphorylation of endogenous MuSK in C2C12 myotubes was diminished in the absence of Abl kinase activity (**Fig. 5d**), indicating that reciprocal tyrosine phosphorylation of MuSK by Abl kinase may occur under physiologic conditions. It should be noted that although tyrosine phosphorylation of RTKs is often attributed solely

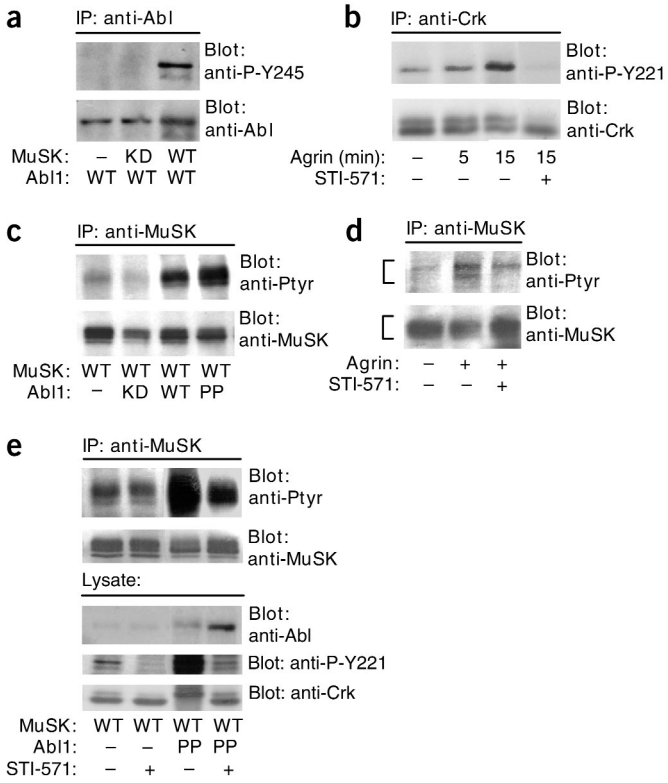

Figure 5 Abl kinases and MuSK effect reciprocal tyrosine phosphorylation. (**a**) Immunoblot of tyrosine-phosphorylated (Y245) and total Abl1 in immunoprecipitates from lysates of HEK cells expressing WT Abl1 with KD MuSK or WT MuSK. (**b**) Immunoblot of tyrosine-phosphorylated (Y221) and total endogenous Crk, an Abl kinase–specific protein substrate, in immunoprecipitates from C2C12 myotubes stimulated with agrin (10 nM) for times indicated and treated with STI-571 (10 μm) or vehicle control. (**c**) Immunoblot of tyrosine-phosphorylated and total MuSK in immunoprecipitates from lysates of HEK cells coexpressing WT MuSK and KD Abl1, WT Abl1 or constitutively active Abl1 (Abl-PP; ref. 41). (**d**) Immunoblot of tyrosine-phosphorylated and total endogenous MuSK in immunoprecipitates from lysates of C2C12 myotubes stimulated with agrin (1 nM) or vehicle control for 20 min in the presence of STI-571 (10 μM) or vehicle control. Note loss of the agrin-induced tyrosine-phosphorylated MuSK doublet after STI-571 addition. (**e**) Tyrosine-phosphorylated and total WT MuSK in the presence or absence of Abl1-PP after treatment with STI-571 (10 μM) or vehicle control for the final 8 h of a 72-h transient overexpression protocol, as detected by immunoprecipitation and immunoblotting. Phospho-Crk (Y221) blotting of identical lysates provided an internal control for STI-571 activity and Abl family kinase inhibition. Note that Crk protein migrates as a doublet; the slower-migrating band corresponds to the tyrosine-phosphorylated form that collapses into the faster-migrating band upon Abl kinase inhibition. Similar results were obtained in three (**a**,**d**,**e**) or six (**b**,**c**) independent experiments.

to autocatalytic activity, NRTK-mediated phosphorylation and activation of such receptors is well documented[44]. Yet in order to confirm that STI-571 was not directly inhibiting MuSK autocatalytic activity, we tested the effect of STI-571 on MuSK tyrosine phosphorylation in the context of either overexpression of MuSK alone or coexpression of MuSK with Abl1. These conditions favored either autophosphorylation of MuSK or transphosphorylation of MuSK by Abl1, respectively. Importantly, STI-571 did not inhibit autophosphorylation of MuSK upon overexpression of this RTK alone in HEK cells but did inhibit transphosphorylation of MuSK in cells coexpressing Abl1 kinase (**Fig. 5e**). This, combined with the failure of STI-571 to inhibit MuSK catalytic activity directly *in vitro* (**Fig. 3e**), indicates that the decrease in agrin-stimulated MuSK phosphorylation observed in the presence of STI-571 does not stem from direct inhibition of MuSK but rather from direct inhibition of Abl kinases and the resultant interruption of an Abl kinase–MuSK signal amplification loop (see **Supplementary Fig. 1** online).

Given that Abl kinases and MuSK interact functionally, we sought to determine whether they interact physically. We expressed Abl1 alone or coexpressed Abl1 and kinase-defective or wild-type MuSK and assayed for the presence of Abl1 in anti-MuSK immunoprecipitates. Abl1 was immunoprecipitated specifically in the presence of wild-type MuSK, demonstrating that the two form a stable complex in cells (**Fig. 6a**). This interaction required an active MuSK catalytic domain, indicating that Abl kinases may bind preferentially to MuSK that has attained an open, active conformation[8], as induced by agrin engagement *in vivo*. Thus, we next tested whether agrin stimulation could induce enhanced complex formation between endogenous Abl kinases and MuSK. Indeed, agrin stimulation of C2C12 myotubes for as few as 5 min produced an enhanced, stable interaction between endogenous Abl kinase and endogenous MuSK (**Fig. 6b**). We conclude that the reciprocal increases in tyrosine phosphorylation observed above with Abl1 and MuSK stem from an inducible kinase–substrate relationship and that Abl kinase involvement in the agrin and MuSK signaling cascade is both early and direct.

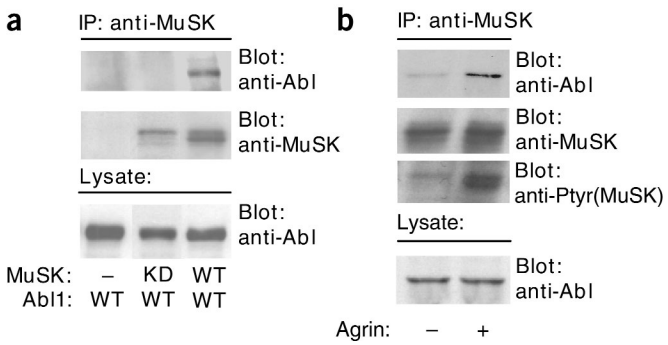

Figure 6 Abl kinases are recruited to the MuSK signaling complex. (**a**) Immunoblot of Abl1 and MuSK in anti-MuSK immunoprecipitates from lysates of HEK cells expressing WT Abl1 alone or WT Abl1 with either KD or WT MuSK. Similar results were obtained in six independent experiments. (**b**) Immunoblot of endogenous Abl kinases in anti-MuSK immunoprecipitates from lysates of C2C12 myotubes stimulated with agrin (10 nM) or vehicle control for 5 min. Similar results were obtained in three independent experiments.

DISCUSSION

Our data show that Abl kinases are critical mediators of postsynaptic assembly at the NMJ. Previous work shows that agrin activates an undefined tyrosine kinase signaling cascade downstream of MuSK that is required for AChR clustering[9]. Although Src kinases associate with the AChR complex, their activity is dispensable for cluster formation[11]. In contrast, our data indicate that Abl kinases provide a specific tyrosine kinase activity downstream of the MuSK receptor that is required for agrin-induced AChR clustering. Moreover, we find that Abl kinases and MuSK are capable of reciprocal tyrosine phosphorylation and complex formation upon agrin engagement;

this has multiple mechanistic implications for postsynaptic assembly at the NMJ. First, phosphorylation of MuSK by Abl kinases could result in the formation of additional phosphotyrosine binding sites for adaptor proteins and thus augment the formation of a larger signaling complex. Second, phosphorylation of MuSK by Abl kinases could increase MuSK catalytic activity and thus facilitate activation of downstream targets, including Abl kinases themselves. Third, phosphorylation of Abl kinases by MuSK could increase Abl kinase catalytic activity leading to phosphorylation of known synaptic components and new effectors of cytoskeletal regulation. Fourth, recruitment of Abl kinases to MuSK could concentrate the intrinsic actin bundling activity of Abl kinases at sites of postsynaptic assembly.

In fact, the dual kinase and actin-regulatory activities of Abl1 and Abl2 make them uniquely suited to the regulation of synaptic architecture. We propose a model in which Abl kinases amplify initial signaling and receptor clustering through tyrosine phosphorylation and stabilize clusters through induction of a synaptic actin scaffold (**Supplementary Fig. 1**). With regard to the latter, both the small GTPases Rac and Cdc42 and their effector Pak have been identified as downstream targets of agrin stimulation[12,13]. It has been proposed that Rac, Cdc42 and Pak constitute a cytoskeletal regulatory module that augments AChR clustering[12,13]. Genetic data in *D. melanogaster* have placed D-Abl upstream of Trio, a guanine nucleotide exchange factor for Rac and Rho[21,45]. By extension, Abl kinase activation downstream of MuSK may lead to the phosphorylation and activation of Rac and/or Cdc42 guanine nucleotide exchange factors and subsequent activation of Pak and other targets of activated GTPases. It should be noted, however, that inhibition of Abl kinase activity results in a block in agrin-induced AChR clustering that is greater than that reported for inhibition of Pak[14]. Thus, this potential Rac/Cdc42/Pak module probably represents only one arm of a multipronged signaling cascade, emanating from Abl kinases, that regulates both tyrosine kinase signal amplification and cytoskeletal reorganization downstream of agrin and MuSK.

Abl2$^{-/-}$ (previously *Arg*$^{-/-}$) mice have motor deficits that are consistent with muscle fatigue upon sustained use[24], although the actual defect underlying this phenotype has yet to be determined. Most human cases of congenital myasthenia have been traced to defective neurochemical transmission at a structurally intact NMJ[2]. A subset of patients, however, has now been identified in which the underlying mutations are in rapsyn[46], a postsynaptic protein of 43 kDa that is also absolutely required for AChR clustering[1]. This finding establishes a precedent for NMJ dysfunction as a direct consequence of defective postsynaptic assembly. Future studies will determine whether genetic ablation of Abl family kinases is sufficient to cause NMJ defects and resultant neuropathology.

The findings presented here have broad implications regarding the role of Abl kinases in synapse formation. A wealth of genetic data, primarily from *D. melanogaster*, has supported a model in which Abl kinases function purely presynaptically in neural development, specifically as mediators of axon guidance[16,21,25]. We have used a combination of immunohistochemical and molecular analyses of murine muscle to provide evidence of postsynaptic function for the Abl kinases. This model of Abl-mediated postsynaptic assembly may be extended to the central nervous system, where Abl2, in particular, is highly enriched[24,31]; it is further supported by the recent finding that Abl1 and Abl2 localize to the postsynaptic density in mouse CA1 hippocampal neurons[47]. EphB receptors regulate glutamatergic synapse formation through a direct interaction with the NMDA receptor[48,49]. Abl kinases interact directly with both EphB2 (ref. 37) and the NMDA receptor[50] and thus could mediate EphB-dependent regulation of synapse formation in the CNS.

METHODS

Chemicals and antibodies. We generated Abl2 CT antibodies by injection of rabbits with a peptide from the Abl2 C terminus (DKDRPRRVKPK) that shared no homology with the corresponding Abl1 sequence; we subsequently purified polyclonal antibodies on a peptide affinity column. Generation of Abl1AM9 and Abl2(SH2-SH3) polyclonal antibodies was as previously described[24,32]. We obtained the following antibodies from commercial sources: Abl C-19, Abl K-12, MuSK N-19, MuSK C-19, PY20 and PY99 (Santa Cruz); Abl Ab-3 (Oncogene Science); Abl 8E9 (Pharmingen); phospho-Abl Y245 and phospho-CrkII Y221 (Cell Signaling Technology); anti-rMuSK (R&D Systems); 4G10 (Upstate Biotechnology); Neurofilament (Sigma); Synaptophysin (Zymed) and Crk mAb (Transduction Laboratories).

Immunohistochemistry. Mouse tibialis anterior or gastrocnemius muscle was flash-frozen in O.C.T. compound (Tissue-Tek) and cut into 10-μm sections on a cryostat. Sections were washed in 1× PBS before staining to remove O.C.T. compound and antibody was applied to unfixed sections in 2% BSA/5% normal goat serum at the following dilutions: Abl2 CT, 1:100; Abl C-19, 1:200; Abl2(SH2-SH3), 1:100; NF, 1:1,000; synaptophysin, 1:2,000; cy3-anti-rabbit secondary antibody, 1:2,000 (Jackson Laboratories). BTX (Alexa Fluor 488; R&D Systems) was used at 1:5,000. We viewed samples on a Zeiss Axioskop 2 Plus with Axiocam and Axiovision software. When indicated, primary antibody was pre-incubated for 1 h with peptide antigen and then applied to samples as above. We fixed C2C12 myotube cultures in 4% paraformaldehyde for 10 min and then stained and imaged them as above, except for the addition of 0.2% Triton X-100 to antibody diluent.

Denervation experiments. Denervation experiments comprised unilateral transection of the sciatic nerve in ketamine- and xylazine-anesthetized mice. We harvested denervated gastrocnemius muscle and contralateral innervated control muscle 5 d post-operation and confirmed denervation by observation of flaccid paralysis and atrophy in the affected limb and muscle, respectively, as well as by staining for axonal markers. Approval for animal experiments was given by the Duke University Institutional Animal Care and Use Committee.

Cell culture, stimulation and transfection. C2C12 myoblasts were passaged in DMEM + 20% fetal bovine serum and differentiated to myotubes in DMEM + 2% horse serum, per standard protocol. After 2–3 d of differentiation, myotubes were stimulated with recombinant agrin (R&D Systems) in the presence or absence of STI-571 for the indicated times, and then fixed and stained as above. Alternatively, cultures stimulated as above were lysed at varying times and used for immunoprecipitation. When indicated, we transfected C2C12 myoblast cultures with kinase-defective (K290R; ref. 19) or wild-type murine *Abl1* in pSRα using Fugene 6 reagent (Roche) as described[7]. HEK cells were passaged in DMEM/F12 + 10% fetal bovine serum and transfected with Fugene 6 reagent. Expression vectors encoding wild-type and kinase-defective rat *MuSK* were as previously described[8], as was constitutively active Abl1 (*Abl-PP*)[41]. We transfected 293T cells with wild-type *Abl1* in pSRα by the calcium-phosphate method. *Abl1*$^{-/-}$*Abl2*$^{-/-}$ fibroblasts were reconstituted with wild-type *Abl2* as previously described[19].

Lysate preparation and immunoblotting. We generated tissue lysates from brain and muscle by Polytron and Dounce homogenization of mouse tissue in 2× electrophoresis sample buffer (without bromophenol blue) followed by centrifugation to remove the insoluble fraction. We determined the protein concentration by BCA assay and analyzed lysates by SDS-PAGE and immunoblotting. HEK, 293T, fibroblast and C2C12 cells were lysed in Abl lysis buffer (ALB: 50 mM HEPES, pH 7.0; 150 mM NaCl; 10% glycerol; 1% Triton X-100; 1.5 mM $MgCl_2$; 1 mM EGTA and protease and phosphatase inhibitors). Abl kinases were immunoprecipitated with Ab-3, Abl2 CT or K-12 antibodies and blotted with 8E9 (1:1,000) or phospho-Abl Y245 (1:1,000). MuSK was immunoprecipitated with MuSK C-19 and N-19 in combination and blotted with anti-rMuSK (1:100). Alternatively, we probed Abl and MuSK immunoprecipitates with three antiphosphotyrosine antibodies in combination: 4G10, PY20 and PY99 (all 1:1,000).

***In vitro* kinase assay.** *In vitro* kinase assays of endogenous Abl1, Abl2 or MuSK from C2C12 myotubes (from 100 μg of ALB lysate) were carried out as

described[19], using a stringent eight-wash protocol after immunoprecipitation, including two 0.5-M NaCl washes and two SDS-RIPA buffer washes. The use of enolase as an *in vitro* MuSK substrate was previously described[10].

Note: Supplementary information is available on the Nature Neuroscience website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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